Regulation of the Human MutYH DNA Glycosylase by Ubiquitination

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TO MY FAMILY

- my parents and my wonderful husband -

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1. Summary

It is of pivotal importance for every organism to maintain the genomic stability since mutations in the genome can lead to the onset of many kinds of diseases like cancer. The DNA is exposed every day to a high amount of oxidative agents derived from endogenous as well as exogenous sources. One of the best-characterized and most abundant DNA lesion arising from oxidative stress is 7,8-dihydro-8-oxoguanine (8-oxo-G). The mutagenic potential of this lesion is given by its ability to form a Hoogsteen base pair with adenine (A). If the 8-oxo-G is not removed before the cell undergoes replication replicative DNA polymerases preferentially incorporate the wrong A instead of the correct C opposite the lesion. Consequently, that can lead to the formation of G:C->T:A transversion mutations – a frequently found lesion in different types of cancer. To counteract the deleterious potential of 8-oxo-G nature has evolved pathways to repair 8-oxo-G lesions. The MutY homologue DNA glycosylase (MutYH) recognizes and removes an A mispaired to 8-oxo-G and subsequently the nicked template can be correctly bypassed by DNA Pol λ. The recruitment of DNA Pol λ to chromatin was shown to be modulated by interplay of phosphorylation and ubiquitination. Until now it was not clear by which mechanisms MutYH is regulated.

In the present thesis work it is shown that MutYH is ubiquitinated by the E3 ligase Mule. Ubiquitination of MutYH targets the protein for proteasomal degradation but also regulates its subcellular localization. An ubiquitination deficient mutant of MutYH is predominantly bound to chromatin with respect to the wildtype. Consistent with its role in base excision repair (BER) the mutation frequency in cells with decreased MutYH levels is elevated upon exposure to $KBrO_3$, while cells with increased levels of MutYH are able to cope better with oxidative damage. Taken together the tight regulation of MutYH is of great importance to guarantee the correct and fast repair of 8-oxo-G lesions since already small differences in the protein level can have deleterious implications for the genomic stability.

2. Zusammenfassung

Es ist für jeden Organismus von größter Bedeutung die genomische Stabilität zu erhalten, da bereits einige wenige Mutationen im Erbmaterial zu Erkrankungen wie Krebs führen können. Die DNA ist jeden Tag einer großen Menge an oxidierenden Substanzen ausgesetzt, die sowohl endogenen als auch exogenen Ursprungs sein können. Eine der am besten untersuchten und am häufigsten vorkommenden DNA Schädigungen ist 7,8-dihydro-8-Oxoguanine (8-oxo-G), das meistens durch oxidativen Stress verursacht wird. Diese Schädigung führt besonders häufig zu Mutationen, da sie in der Lage ist, ein stabiles Hoogsteen Basenpaar mit Adenin (A) zu bilden. Die Wahrscheinlichkeit, dass das falsche A als Gegenstück zu 8-oxo-G eingefügt wird ist sehr hoch, wenn die DNA Schädigung nicht vor der Zellteilung entfernt wird, da die replikativen DNA Polymerasen bevorzugt A einfügen. Als Konsequenz werden G:C->T:A Transversionsmutationen generiert, die sehr häufig bei Krebs-Patienten identifiziert wurden. Um das Auftreten dieser Mutationen zu verhindern hat die Natur verschiedene Reparaturmechanismen entwickelt. Die DNA Glycosylase MutYH erkennt und entfernt ein A, das fälschlicherweise gegenüber von 8-oxo-G integriert wurde. Der daraus resultierende, geschnittene DNA Strang wird anschließend von der DNA Pol λ korrekt wieder aufgefüllt. Die Rekrutierung von DNA Pol λ an das Chromatin wird durch ein Zusammenspiel von Ubiquitinierung und Phosphorylierung reguliert. Bisher war es allerdings nicht bekannt wie die Regulation von MutYH erfolgt.

In der hier vorgelegten Dissertationsarbeit wird gezeigt, dass MutYH von der E3 Ligase Mule ubiquitiniert wird. Die Ubiquitinierung von MutYH führt einerseits zu der proteasomalen Degradierung und reguliert andererseits auch die Lokalisierung von MutYH in der Zelle. Im Vergleich zum Wildtyp liegt eine Ubiquitinierungs-defiziente Mutante von MutYH vorwiegend ans Chromatin gebunden vor. In Übereinstimmung mit der Rolle, die MutYH in der Basen Excisionsreparatur (BER) spielt, kann gezeigt werden, dass Zellen in Abhängigkeit von der MutYH Konzentration besser oder schlechter mit oxidativem Stress umgehen können. Zellen mit niedrigerem MutYH Level zeigen eine höhere Mutationsfrequenz, wohingegen Zellen mit mehr MutYH weniger Mutationen aufweisen. Zusammenfassend kann gesagt werden, dass eine sehr genaue Regulierung von MutYH notwendig ist um die Reparatur von 8-oxo-G schnell und korrekt zu gewährleisten. Schon geringe Änderungen des Protein-Spiegels können schwerwiegende Konsequenzen für die Stabilität des Genoms haben.

3. Introduction

3.1. DNA damage

DNA damage is the source of many types of cancer and diseases related with aging. Every organism is exposed to high numbers of different factors attacking the integrity of DNA, either arising from endogenous or exogenous sources. Physical and chemical agents can cause DNA damage as well as spontaneous reactions, mostly hydrolysis, leading to deamination and formation of abasic sites (1,2). A substantial amount of reactive oxygen species (ROS) is generated endogenously as a byproduct of oxidative respiration, thus the mitochondria are the main producers of ROS. Electrons leaking from the electron transport chain can directly go over to oxygen molecules (3) and cause thereby the formation of very reactive oxygen radicals.

As soon as ROS are produced they can react with many macromolecules like proteins, lipids and DNA molecules and cause oxidative damage (4,5). The reaction of oxygen radicals with DNA leads to the formation of different DNA base products (Figure 1). Among those 7,8-dihydro-8-oxoguanine (8-oxo-G) is the most abundant, but also the best-characterized DNA lesion.

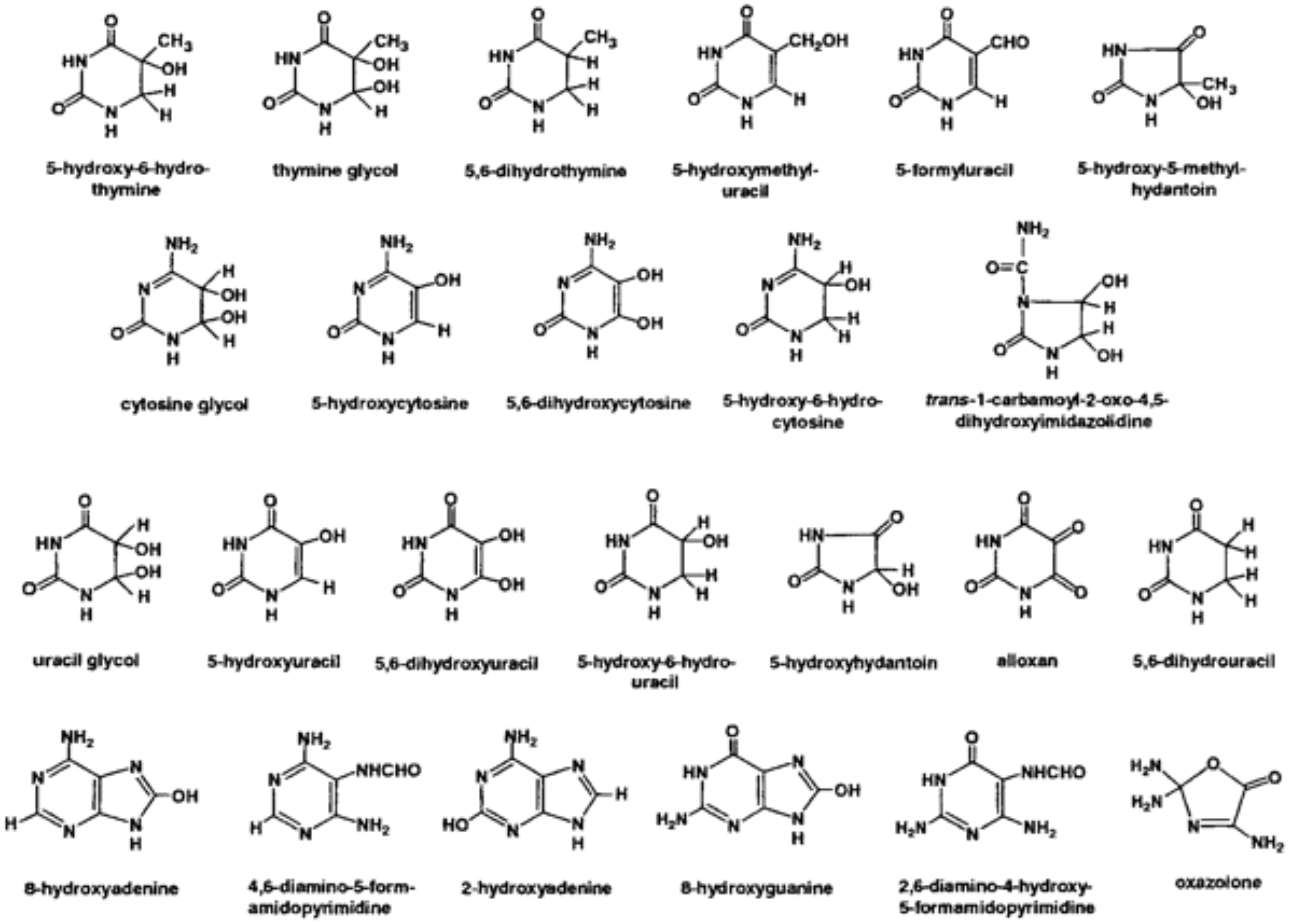

Figure 1: DNA base products.
Reproduced from (4).

Taken together the amount of DNA damage is estimated to be about 1000-7000 lesions per cell and day (6). DNA lesions can lead to the generation of mutations or chromosomal aberrations that are related to an increased risk of cancer.

Especially the base guanine (G) is affected by the constant exposure of genomic DNA to oxidation reactions. Guanine has the lowest redox potential making it very susceptible to oxidative stress (7). Therefore the formation of 8-oxo-G occurs often under oxidative conditions. The pairing of the lesion with adenine (A) causes G:C->T:A transversion mutations if left unrepaired (8). In case these mutations occur in proto-oncogenes it may cause cancer development. Greenman *et al.* analyzed for the first time the appearance of somatic point mutations in different cancer tissues (9). They found the G:C->T:A transversions as the most prevalent mutations in the coding exons of 518 protein kinase genes in 210 human tissue samples derived from lung, breast, ovarian, gastric and colorectal cancers. Strikingly a study aiming to sequence small-cell lung cancer cells for somatic mutations came to the same conclusion. Pleasance *et al.* identified G:C->T:A transversion mutations to make one third of all observed somatic muta-

tions (10). Accordingly, in the analysis of an individual cancer genome from melanoma cells the G:C->T:A transversion mutations were found to be the second most prevalent (11). Thus, it is of pivotal importance for the human body to control and repair DNA damages as fast and accurate as possible to avoid the onset of diseases like cancer.

3.2. Base excision repair (BER)

3.2.1. Short- and long-patch BER

Nature has evolved a large number of repair processes to mitigate the deleterious potential of DNA lesions. 8-oxo-G is in the first place repaired by the short- (SP-BER) or long-patch BER (LP-BER) (Figure 2). DNA repair is initiated by the action of DNA glycosylases, removing the lesion by incision of the N-glycosylic bond thereby creating an apurinic/apyrimidinic (AP) site. This site is further targeted by the apurinic/apyrimidinic endonuclease 1 (APE1) that generates a single strand break with a 3`-hydroxyl and a 5`deoxyribose-5`-phosphate (5`dRP) termini (12). This intermediate is further cleaved by the dRP lyase activity of DNA Pol β, causing the formation of a one-nucleotide gap. The subsequent repair processes differ from each other regarding the size of the repair patch. During the SP-BER just one nucleotide is replaced by DNA Pol β and the nick is finally sealed by the DNA ligase III/X-ray repair cross complementing 1 protein (XRCC1) (13,14).

The LP-BER requires a strand displacement activity since 2-12 nucleotides are removed (15,16). The executing Pols are most likely DNA Pol β that incorporates the first nucleotide (17) and DNA Pol ε or DNA Pol δ that perform the following elongation steps. To locate the Pols at the site of damage additional factors are required. Replication factor C (RF-C) loads proliferating cell nuclear antigen (PCNA) onto the DNA and PCNA then acts as sliding clamp for the Pols. Finally the flap endonuclease 1 (Fen 1) excises the displaced nucleotides and the remaining nick is sealed by DNA ligase I (18,19).

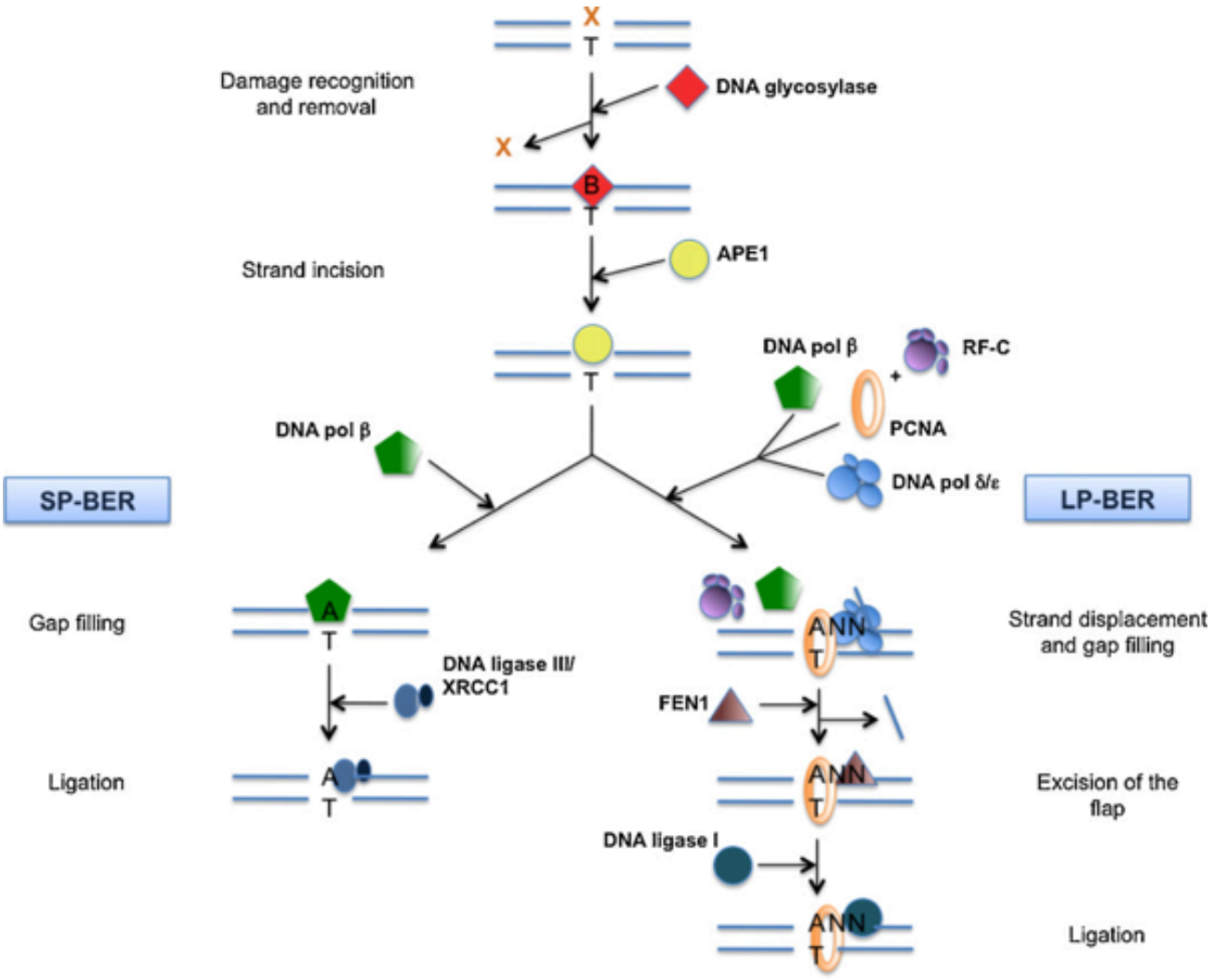

Figure 2: Short- and long-patch base excision repair.
Reproduced from (20). For details see text.

3.2.2. The 8-oxo-G problem

The DNA is exposed to a variety of endogenous and exogenous sources of oxidative stress. Among the four bases especially G is vulnerable to oxidation due to its very low redox potential (7). The oxidation of G leads to the addition of an oxo-group to the carbon located at position 8 (C8) and a hydrogen to the nitrogen at position 7 (N7) (Figure 3). The deleterious potential of 8-oxo-G can be attributed to its ability to mimic a thymidine (T). In the *syn* conformation 8-oxo-G is able to form a Hoogsteen base pair with A that resembles a normal Watson-Crick base pair (21). In contrast to that the pairing with C would require the *anti* conformation and subsequently lead to a template distortion.

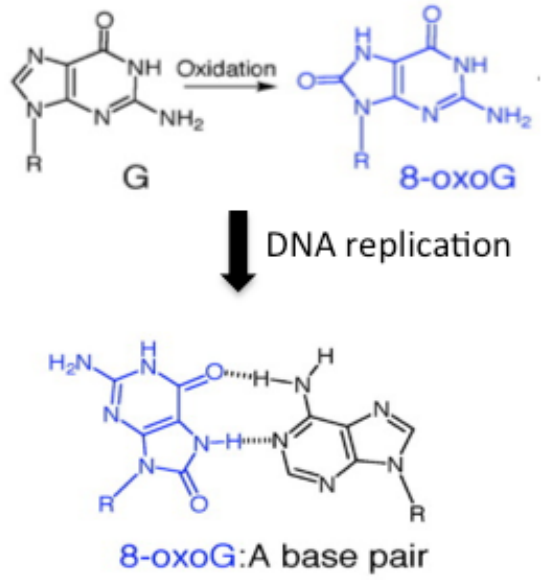

Figure 3. Formation of 8-oxo-G and pairing with A.
Modified from (22). For details see text.

Therefore almost all of the so far tested Pols tend to incorporate rather the wrong A than the correct C opposite the lesion (21,23-29) leading to the onset of G:C ->T:A transversion mutations. 8-oxo-G was shown to arise approximately 10^3 times per day in normal cells and up to 10^5 times in cancer cells (30). Given its high prevalence in cancers and its mutagenic potential it is of high importance for the human body to have effective repair mechanisms to counteract 8-oxo-G.

Most organisms have, in analogy to bacteria evolved the so-called “GO-system” to counteract 8-oxo-G (31). It is a three-enzyme-based system comprising of two DNA glycosylases from the BER and one dGTPase. The eukaryotic homologues are the 8-oxo-G dGTPase (MTH1) (32) and the MutY homologue DNA glycosylase (MutYH) (33). In contrast to these two enzymes is the 8-oxo-G DNA glycosylase (Ogg1) not related to the bacterial Fpg, but is rather a functional orthologue (34,35). Ogg1 recognizes and removes the 8-oxo-G lesion generating a substrate for the subsequent BER. MTH1 hydrolyzes free 8-oxo-dGTP thereby eliminating it from the nucleotide pool and preventing its incorporation into DNA. The action of MutYH is required as soon as 8-oxo-G lesions are not removed before the replication cycle starts. In contrast to Ogg1, MutYH is directed to the new synthesized daughter strand and recognizes and removes an A mispaired to the lesion.

3.2.3. The MutYH initiated long-patch base excision repair

For a long time 8-oxo-G:A mispairs were thought to be repaired by the conventional LP-BER. The DNA Pols δ and ε, involved in LP-BER, (36-38) are highly error prone in the correct bypass of 8-oxo-G. They incorporate in 30-50% the wrong A opposite 8-oxo-G instead of the correct C (39). Thus another repair pathway is needed to overcome the deleterious potential of 8-oxo-G. Van Loon *et al.* found a MutYH initiated LP-BER pathway involving the action of DNA Pol λ (Figure 4) (40).

MutYH is recruited by the interaction with PCNA to the site of the lesion (1), recognizes the 8-oxo-G and excises the A (2) that got incorporated erroneously during replication. APE1, another PCNA interacting protein (41), is also bound to the DNA and generates a nicked strand (3). Next DNA Pol λ inserts with the help of replication protein A (RP-A) and PCNA the correct C opposite the lesion (4) and an additional one-nucleotide flap. After dissociation of DNA Pol λ Fen1 is recruited through interaction with PCNA (42,43) and removes the overhanging nucleotide (5). Finally PNCA mediates the binding of DNA ligase 1 (44,45) that seals the nick (6).

An alternative scenario can take place when DNA Pol λ incorporates the wrong A opposite the lesion (A). In this case an inaccurate SP-BER is initiated. After DNA Pol λ and RP-A dissociated from the repair intermediate the DNA ligaseIII/XRCC1 complex is recruited from PCNA (46) to ligate the nick (B). This pathway leads to the formation of an 8-oxo-G:A mispair (C/D) that can serve again as substrate for the MutYH mediated LP-BER giving the cell another chance to repair the lesion correctly thus preventing mutations.

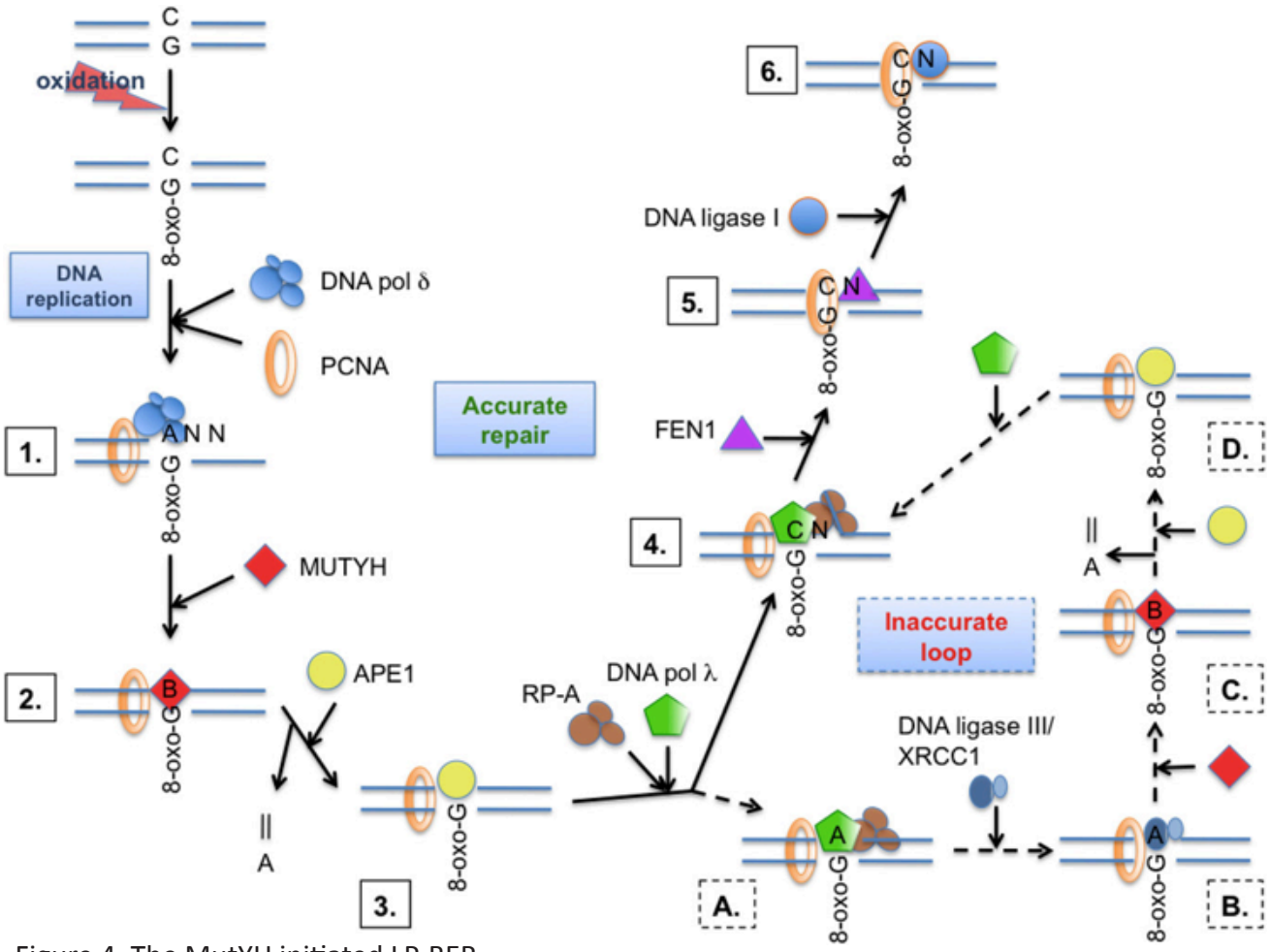

Figure 4. The MutYH initiated LP-BER.
Reproduced from (40). For details see text.

3.3. The MutYH DNA glycosylase

MutYH is a monofunctional glycosylase, meaning it lacks an additional AP lyase activity (47). The glycosylase belongs to the family of helix-hairpin-helix (HhH) glycosylases and the encoding gene is located at the short arm of chromosome 1 (1p32.1 - p34.3). The catalytic domain is located in the N-terminal part of the protein containing an [4FE-4S] iron sulfur cluster (48,49), while the C-terminus consists of the MutT-like domain (23) that was shown to have an important role in substrate recognition. The 8-oxo-G lesion is recognized and bound mainly by the MutT-like domain (50) and the mispaired A is flipped out into a pocket of the active site where the glycosidic bond is cleaved (51,52).

MutYH is spliced in a mitochondrial (53) and a nuclear form (54) since the sequence of the protein contains a nuclear and a mitochondrial localization signal. The exact number of different splice variants is still under debate and discussed controversial (55-59).

MutYH interacts with many proteins involved in DNA damage repair, replication and cell cycle checkpoints like DNA Pol λ (40), RP-A (60), the 9-1-1 complex (61,62), PCNA

(60,63), MSH6 (64), ATR (65) and APE1 (60) (Figure 5). This interaction can either stimulate the glycosylase activity of MutYH (62,64,66) or can regulate the recruitment to chromatin (67).

Al-Tassan *et al.* identified germline mutations in patients suffering from colorectal carcinoma and adenoma (68). These mutations impaired the glycosylase activity dramatically (69,70) and caused thereby a massive increase in mutations leading to the onset of cancer. Further investigations identified more patients carrying MutYH mutations, like missense and in-frame mutations as well as mutations leading to different splicing constructs (Figure 5) (71). Some of them were shown to enhance the mutator phenotype of human cells under conditions of oxidative stress (72). However, many mutations identified in MutYH associated polyposis (MAP) patients are not characterized so far and their role in the development of cancer remains elusive.

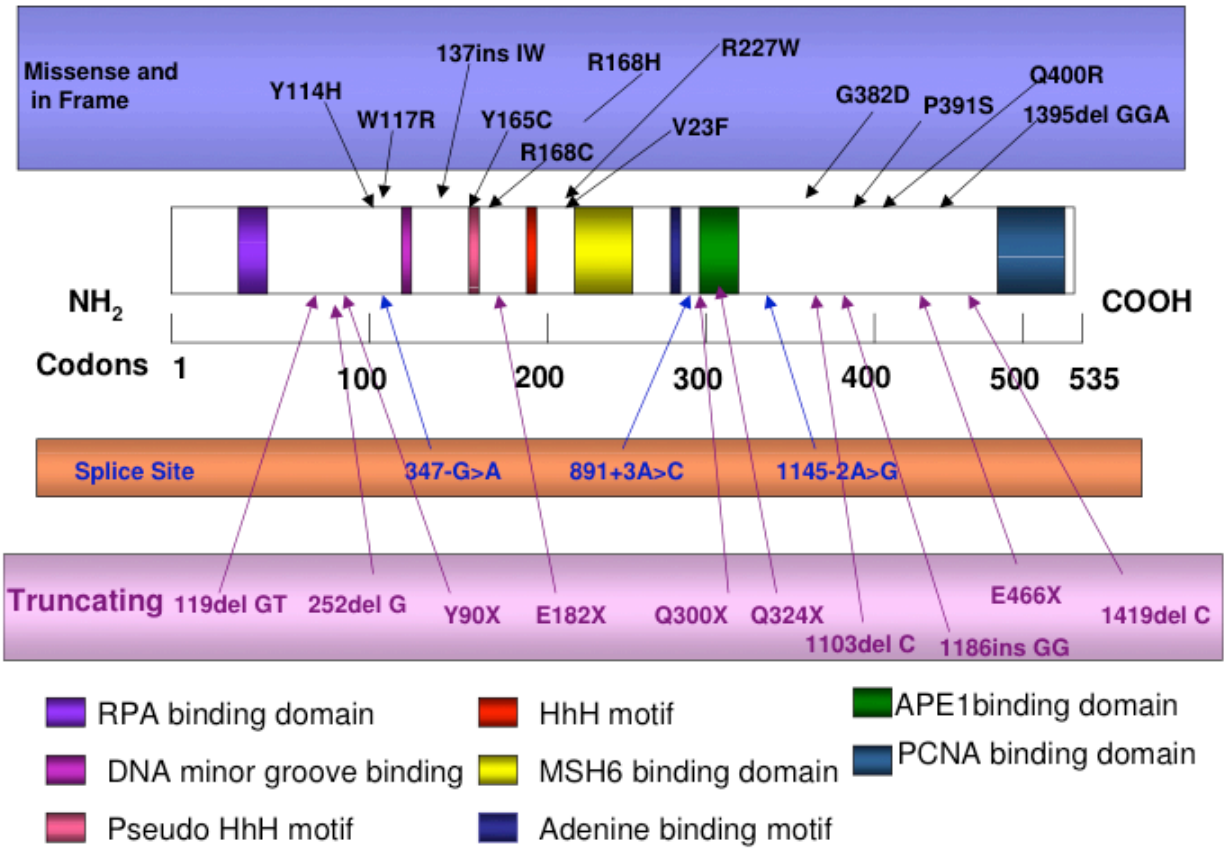

Figure 5: Diagram of MutYH including three typical types of mutations identified in patients suffering from MAP.
Reproduced from (73,74). For details see text.

Only a few reports are published concerning the regulation of MutYH. The protein levels were shown to peak in S-phase (58), what is in accordance with its predicted

role in replication-coupled repair (75). Apart from publications reporting MutYH to be phosphorylated nothing is known about posttranslational modifications (PTM) of MutYH. Depressed phosphorylation levels of MutYH caused a decrease in the catalytic activity and thereby an enhanced mutation frequency in colorectal cancer cells (76,77). Further *in vitro* studies also confirmed the importance of phosphorylation for the glycosylase activity of MutYH (78,79).

In the following review *"MutYH DNA glycosylase: the rationale for removing undamaged bases from DNA"*, all aspects of MutYH are discussed in more detail.

3.4. Review : “MutYH DNA glycosylase: the rationale for removing undamaged bases from DNA”

Enni Markkanen, Julia Dorn and Ulrich Hübscher
Frontiers in genetics, 2013, 4, 18.

The following review, which I co-authored, is a summary of all aspects concerning MutY and MutYH DNA glycosylases.

An update of the literature published since February 2013 is summarized following the original publication.

frontiers in GENETICS

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MUTYH DNA glycosylase: the rationale for removing undamaged bases from the DNA

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Maintenance of genetic stability is crucial for all organisms in order to avoid the onset of deleterious diseases such as cancer. One of the many proveniences of DNA base damage in mammalian cells is oxidative stress, arising from a variety of endogenous and exogenous sources, generating highly mutagenic oxidative DNA lesions. One of the best characterized oxidative DNA lesion is 7,8-dihydro-8-oxoguanine (8-oxo-G), which can give rise to base substitution mutations (also known as point mutations). This mutagenicity is due to the miscoding potential of 8-oxo-G that instructs most DNA polymerases (pols) to preferentially insert an Adenine (A) opposite 8-oxo-G instead of the appropriate Cytosine (C). If left unrepaired, such A:8-oxo-G mispairs can give rise to CG→AT transversion mutations. A:8-oxo-G mispairs are proficiently recognized by the MutY glycosylase homologue (MUTYH). MUTYH can remove the mispaired A from an A:8-oxo-G, giving way to the canonical base-excision repair (BER) that ultimately restores undamaged Guanine (G). The importance of this MUTYH-initiated pathway is illustrated by the fact that biallelic mutations in the *MUTYH* gene are associated with a hereditary colorectal cancer syndrome termed MUTYH-associated polyposis (MAP). In this review, we will focus on MUTYH, from its discovery to the most recent data regarding its cellular roles and interaction partners. We discuss the involvement of the MUTYH protein in the A:8-oxo-G BER pathway acting together with pol λ, the pol that can faithfully incorporate C opposite 8-oxo-G and thus bypass this lesion in a correct manner. We also outline the current knowledge about the regulation of MUTYH itself and the A:8-oxo-G repair pathway by posttranslational modifications (PTM). Finally, to achieve a clearer overview of the literature, we will briefly touch on the rather confusing MUTYH nomenclature. In short, MUTYH is a unique DNA glycosylase that catalyzes the excision of an undamaged base from DNA.

Keywords: MUTYH, MUTYH-associated polyposis (MAP), MYH, mutY, DNA polymerase beta and lambda, base-excision repair (BER), DNA glycosylases, 8-oxo-guanine

INTRODUCTION

Cellular DNA is constantly under attack of damaging agents, such as reactive oxygen species (ROS), that derive from a multitude of exogenous and endogenous sources (reviewed in Van Loon et al., 2010). One of the main consequences of ROS impact on DNA is the formation of 8-oxo-G, a frequent DNA lesion estimated to arise around 1000–7000 times per cell per day (Collins, 1999; European Standards Committee on Oxidative DNA Damage (ESCODD), 2003; Gedik and Collins, 2005; Friedberg, 2006). To counteract this heavy burden of 8-oxo-G lesions, a multi-component system involving a plethora of enzymes has evolved both in bacteria and mammals. 8-oxo-dGTP, which arises upon oxidation of the nucleotide pool, is hydrolyzed by the enzymes MutT/MTH1, which therefore prevent incorporation of 8-oxo-dGTP into nascent DNA. When a C:G base pair is oxidized to C:8-oxo-G, the enzyme Fpg (also known as MutM)/OGG can catalyze the removal of 8-oxo-G from these base pairs. Furthermore, other proteins such as the mismatch-repair pathway component MutS/MSH2, or the Nei endonuclease VIII/NEIL1 and NEIL2 have been shown to protect the genome from the mutagenic consequences of 8-oxo-G damage. Finally, A:8-oxo-G base pairs are a substrate for MutY/MUTYH, which is the protein in the focus of this review. Information on the contribution of all of the other factors to genetic stability can be found in these detailed reviews (Lu et al., 2006a; Tsuzuki et al., 2007).

In the *syn* conformation, 8-oxo-G functionally mimics the base pairing properties of a Thymine (T), which leads to the formation of stable A(*anti*):8-oxo-G(*syn*) Hoogsteen base pairs (David et al., 2007). Due to this particular behavior of 8-oxo-G, most pols often bypass 8-oxo-G lesions inaccurately by incorrectly inserting an A instead of the correct C, therefore giving rise to A:8-oxo-G mismatches (Maga et al., 2007). If these A:8-oxo-G mismatches are not repaired before the next round of replication, they can generate CG→AT transversion mutations that have the potential to transform cells and lead to cancer (Greenman et al., 2007). Oxidative damage to C:G base pairs in DNA leads to the generation of C:8-oxo-G base pairs. The majority of 8-oxo-G from these base pairs is recognized and removed from the genome by the OGG1 DNA glycosylase, which initiates a canonical short-patch base-excision repair (SP-BER) pathway

involving apurinic endonuclease 1 (APE1), pol β, XRCC1, and DNA ligase III. This results in the restoration of the original C:G base pair [see **Figure 1**, Dianov et al., 1998; Fortini et al., 1999; Pascucci et al., 2002; Fromme et al., 2003 and reviewed in Van Loon et al. (2010)]. However, a problematic situation may arise when the replication fork encounters an 8-oxo-G. Such a scenario can result from either a failure of OGG1 to repair all 8-oxo-G lesions before the start of replication, or from oxidative stress during the S-phase. In contrast to UV-induced lesions, for instance, that present a block to the replicative pols (reviewed in Lehmann, 2002), 8-oxo-G is not considered a blocking lesion *per se* (Shibutani et al., 1991; Mozzherin et al., 1997; Avkin and Livneh, 2002). Nevertheless, it has been found that replicative pols (such as the Klenow fragment of *E. coli* pol I, calf thymus pol α and pol δ) show transient inhibition of chain extension 3′ to 8-oxo-G and extend promutagenic A:8-oxo-G base pairs more efficiently than the correct C:8-oxo-G base pairs (Shibutani et al., 1991; Einolf and Guengerich, 2001). Also, human pol δ has been demonstrated to stall at sites of 8-oxo-G lesions (Fazlieva et al., 2009). Very recently, we have proposed that a switch between the replicative pol δ and the repair pol λ promotes the correct bypass of 8-oxo-G lesions during replication (Markkanen et al., 2012a). Nevertheless, oxidative stress in context of DNA replication can result in the generation of A:8-oxo-G mispairs, which are

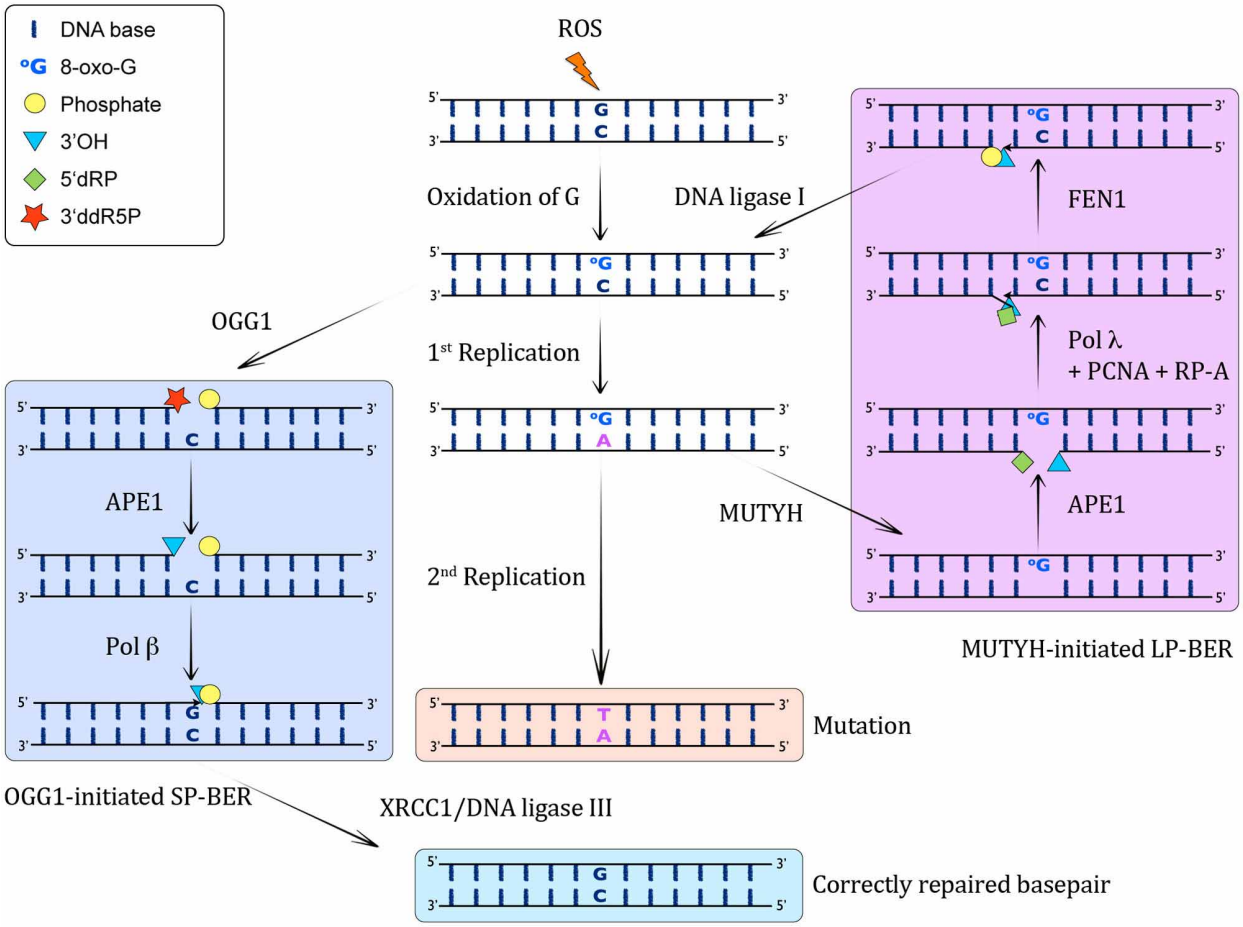

FIGURE 1 | MUTYH-initiated BER of A:8-oxo-G lesions. When ROS attack DNA, they lead to the formation of C:8-oxo-G base pairs through oxidation of G. **Left column:** These can be recognized by OGG1, which excises the 8-oxo-G and incises the resulting AP-site by β-elimination, giving rise to a 3′ddR5P and a 5′P residue. This 3′ sugar phosphate is then removed by APE1, yielding in a 1 nucleotide gap with a 3′OH and a 5′P. Subsequently, pol β catalyzes the insertion of a G opposite the templating C in this SP-BER pathway, and ligation by XRCC1/DNA ligase I leads to restoration of an intact, correctly base-paired double-stranded DNA again. **Middle column:** If the C:8-oxo-G base pairs are not recognized before S-phase by OGG1, or they arise through oxidation in S-phase, the replicative pols will often incorporate a wrong A opposite 8-oxo-G, giving rise to A:8-oxo-G mispairs. If these are not corrected, another round of replication will lead to a CG→AT transversion mutation. **Right column:** The A:8-oxo-G base pairs can be recognized by MUTYH, which catalyzes the excision of the wrong A from opposite 8-oxo-G, leading to the formation of an AP site. This AP site is further processed by APE1, which results in a 1 nt gap with 3′OH and 5′dRP moieties. The incorporation of the correct C opposite 8-oxo-G and one more nucleotide is performed by pol λ in collaboration with the cofactors PCNA and RP-A, thus performing strand displacement of the downstream DNA strand. FEN1 cleaves the 5′ flap, leading to a 5′P moiety, which can be ligated by DNA ligase I to yield an intact C:8-oxo-G containing double-stranded DNA. This C:8-oxo-G is then again substrate for OGG1-mediated removal of 8-oxo-G **(left column)**.

substrates for MUTYH. As a monofunctional DNA glycosylase, MUTYH catalyzes the excision of the A mispaired with 8-oxo-G. Thus, MUTYH is a unique glycosylase as far as it removes an *undamaged* base from *opposite a DNA lesion*, instead of removing the damaged base. The steps following MUTYH-initiated repair of A:8-oxo-G lesions are discussed in more detail in the following. As this review is focused on MUTYH, the interested reader is referred to a detailed excellent review for more information on the cellular DNA glycosylases in general (Jacobs and Schar, 2012).

DISCOVERY

MutY, along with the other 8-oxo-G repair enzymes FpG and MutT, is phylogenetically an ancient protein, emphasizing the importance to cope correctly and efficiently with oxidative damage for living organisms (Jansson et al., 2010). MutY homologues have been identified in many organisms, both in prokaryotes as well as in eukaryotes. They all share the unique function of being able to remove an A that is incorrectly paired with 8-oxo-G, G, C, 5-hydroxyuracil (5-OH-U), or 2-hydroxyadenine (2-OH-A), as specified later on.

DISCOVERY OF MutY IN *E. coli*

The first mutators in *E. coli* strains were described about 60 years ago (Treffers et al., 1954) based on the observation that some strains showed an altered antibiotic resistance. These findings were used to engineer a systematic screening for mutators with certain properties. Nghiem et al. used Lac^- *E. coli* strains transformed with constructs encoding for β-galactosidase, each inactivated by a specific point mutation. When reverted back to Lac^+ the specific base substitution reactivating the β-galactosidase could be identified. A strain with an increase in C:G→A:T transversion mutations revealed the so far not described locus called *mutY* to be responsible for the observed mutator phenotype (Nghiem et al., 1988).

In addition to the *mutY*, another locus, called *mutM*, was found to cause a change from C:G→A:T (Cabrera et al., 1988) when mutated and was later identified to encode the formamidinopyrimidine DNA glycosylase (Fpg) (Michaels et al., 1991). Neither *mutY* nor *mutM* strains showed a very pronounced phenotype on their own, but double mutant strains expressed an extremely high mutation rate (Michaels et al., 1992a). Mutations in *mutY* and *mutM* exclusively enhanced one type of transversion mutation, while neither frameshifts nor deletions were found, in contrast to what had been reported for other mutators (Nghiem et al., 1988).

It had been shown that the correction of A:G mispairs in *E. coli* extracts could occur by two distinct pathways: the methylation-dependent mutHLS mismatch-repair pathway that recognizes a variety of mismatches and repairs the unmethylated DNA strand, and a second methylation-independent mechanism specific to A:G mismatches (Su et al., 1988). Analysis of the second pathway revealed that the *mutY* gene product was involved in this novel DNA repair mechanism (Au et al., 1988). Cells defective in the mutHLS-dependent repair but proficient for *mutY* were still able to prevent C:G→A:T transversion mutations, and the *mutY*-dependent repair was dominant if both pathways were available. The function of the *mutY* gene product was finally elucidated by purification of a protein according to its ability to repair a A:G mismatch. The 36 kDa protein was capable of removing the mispaired base A from dsDNA and rendered the strand sensitive for cleavage by apurinic/apyrimidinic endonucleases at the site of the mismatch (Au et al., 1989). This result further underlined the hypothesis that mutY encoded for a DNA glycosylase, termed MutY, that initiated the repair of A:G mismatches while other mispairs, as for example A:C, were not recognized. Further on, Su et al. showed that MutY, with help of pol I and DNA ligase, was able to restore specifically A:G mismatches to C:G in a sequence independent manner (Su et al., 1988). Cloning and sequencing of the mutY gene finally revealed that it encoded for a 350 amino acids DNA glycosylase that could rescue the mutator phenotype of mutY *E. coli* strains (Michaels et al., 1990).

DISCOVERY OF THE MAMMALIAN MutY HOMOLOG (MUTYH)

The first experiments using cell extracts showed that, in general, humans had a repair mechanism for mismatches similar to those of bacteria preventing the generation of mutations during replication (Holmes et al., 1990; Thomas et al., 1991). The analysis of human HeLa nuclear extracts revealed the existence of two enzyme systems that could nick DNA specifically at sites of mispaired bases (Yeh et al., 1991). One of the identified systems showed a specific substrate recognition, cleaving the DNA at A:G mismatches and could be separated from other enzymes by chromatography. Since this enzyme showed the same substrate specificity as the bacterial MutY, Yeh et al. proposed to have identified its human homologue (Yeh et al., 1991).

The first characterization of a mammalian homologue of MutY was published by McGoldrick et al., who purified an enzyme from calf thymus that was acting on A:G mismatches. Apart from the substrate specificity they described several other features indicating that they had indeed purified a MutY homologue: An AP endonuclease activity was co-purified with the DNA glycosylase and the antibody generated against bacterial MutY recognized a band at the expected size and could inhibit the DNA glycosylase activity of the purified protein (McGoldrick et al., 1995). Based on the finding that CG→AT transversion mutations occur often in different kinds of cancer (Hollstein et al., 1991), the authors already hypothesized that the human MutY homologue might be involved in cancer prevention.

A few years after the characterization of human homologue of the 8-oxo-dGTP hydrolase MutT which removes 8-oxo-dGTP from the nucleotide pool (Sakumi et al., 1993), Slupska et al. succeeded in cloning and sequencing of the human *mutY* gene, termed *MUTYH* (Slupska et al., 1996). By screening different cDNA libraries for amino-acid sequence homologies, they identified a gene that showed 41% identity with the *E. coli mutY*. The gene was 7.1 kb long, contained 15 introns and encoded for a protein of 535 amino acids in length, which was consistent with the size of the protein that had been detected in HeLa cells (McGoldrick et al., 1995). By using *in situ* hybridization they could map the gene on chromosome 1, between *p32.1* and *p34.3*. The current status of knowledge is that the human *MUTYH* gene codes for at least 10 different isoforms of MUTYH protein. There are three major transcripts, α, β, and γ that differ from each other in the 5′ end sequence and are generated through alternative

splicing (Ohtsubo et al., 2000). The transcript α3 was found to be the originally identified MUTYH, but so far it is not entirely clear what the functions of the different isoforms are and to which cell compartment they are localized, as we will discuss below in more detail.

NOMENCLATURE OF MUTYH

Currently, literature referring to the protein product of the mammalian *MUTYH* gene is rather confusing due to a diversity of different synonyms and writing styles that have been used over the last years. The most commonly used names are MUTYH, MutYH, MYH, and hMYH. Here, we propose to uniformly use MUTYH as name for this protein in mammals in order to simplify the literature overview, because of the following reasons. Firstly, *MUTYH* [*MutY h*omolog (*E. coli*)] is the officially approved name for the gene from which MUTYH derives (HUGO Gene Nomenclature Committee). Secondly, the official protein name listed by leading protein databases (UniProtKB, neXtProt, Ensembl, and Reactome) is MUTYH. Thirdly, as the protein derives its name from the bacterial homolog mutY that was discovered first, the logical extension would be the addition of an "H" for "homolog" at the end of the protein name, which also leads to easy recognition of homology between MUTYH and MutY.

FUNCTION OF MutY AND MUTYH

MutY

MutY—substrate specificity

The currently known substrates for MutY and MUTYH are summarized in **Table 1**. Analysis of the substrate specificity for MutY demonstrated that it acts as a glycosylase on A:G, A:8-oxo-G, A:C, and A:8-oxo-A mismatches, always removing the undamaged A from each substrate (Michaels et al., 1992b). Lu et al. further refined the DNA determinants and substrate specificities for the catalytic activity of MutY, using binding and endonuclease assays with a variety of different A-containing mismatches, and concluded that DNA sequences proximal to the mismatch as well as specific functional groups of mismatched bases dictate the recognition and catalysis by MutY (Lu et al., 1995). Moreover, while MutY bound the A:8-oxo-G much tighter than A:G, its activity on A:8-oxo-G was weaker than on A:G mismatches. Bulychev et al. contradicted this notion in a subsequent report stating that A:8-oxo-G appeared to be the natural substrate for MutY, as judged by the specificity constants and the fact that the presence of an 8-oxo-group in G increased significantly the rate of removal of A from all tested substrates (Bulychev et al., 1996). Additionally to A:8-oxo-G, MutY was shown to bind to G:8-oxo-G mismatches as well, and it was capable of removing G from this substrate (Zhang et al., 1998). The sequence context surrounding an A:G mismatch was shown to also significantly influence the catalytic activity of MutY (Sanchez et al., 2003).

8-oxo-G is chemically labile toward further oxidation into guanidinohydantoin (Sp1), spiroiminodihydantoin (Sp2), oxaluric acid, and urea. Delaney et al. investigated the activity of MutY on these lesions by introducing them into single-stranded viral genomes which were replicated in *E. coli* proficient or deficient for MutY (Delaney et al., 2007). These lesions were found to be equally mutagenic in terms of frequency in both genetic backgrounds and to yield similar mutation spectra, suggesting that MutY does not play a role in the excision of these bases. Interestingly Sp1 and Sp2 were more toxic to the cells that were proficient in MutY.

Table 1 | Substrate specificities of the different MutY and MUTYH proteins.

Protein	Base pair substrate	Excised base	References
MutY *E. coli*	A:G	A	Michaels et al., 1992b; Lu et al., 1995; Gogos et al., 1996; Noll et al., 1999; Gu and Lu, 2001
	A:8-oxo-G	A	Michaels et al., 1992b; Lu et al., 1995; Gogos et al., 1996; Noll et al., 1999; Gu and Lu, 2001
	A:C	A	Michaels et al., 1992b
	A:8-oxo-A	A	Michaels et al., 1992b
	2-OH-A:G	2-OH-A	Hashiguchi et al., 2002; Pope and David, 2005
	2-OH-A:8-oxo-G	2-OH-A	Pope and David, 2005
	A:FapyG	A	Wiederholt et al., 2003
	G:8-oxo-G	G	Zhang et al., 1998
MutY *Th. thermophilus*	A:8-oxo-G	A	Back et al., 2006
	A:G	A	Back et al., 2006
	G:8-oxo-G	G	Back et al., 2006
	T:8-oxo-G	T	Back et al., 2006
MUTYH *S. pombe*	G:8-oxo-G	G	Doi et al., 2005
	A:8-oxo-G	A	Doi et al., 2005
MUTYH mouse	A:8-oxo-G	A	Tominaga et al., 2004; Pope and David, 2005
	A:G	A	Pope and David, 2005
	2-OH-A:G	2-OH-A	Pope and David, 2005
	2-OH-A:8-oxo-G	2-OH-A	Pope and David, 2005
MUTYH calf	A:G	A	McGoldrick et al., 1995; Parker et al., 2000
	A:8-oxo-G	A	McGoldrick et al., 1995; Parker et al., 2000
	A:C	A	McGoldrick et al., 1995; Parker et al., 2000
	G:8-oxo-G	G	Parker et al., 2000
	T:8-oxo-G	T	Parker et al., 2000
	C:8-oxo-G	C	Parker et al., 2000
MUTYH human	A:8-oxo-G	A	Slupska et al., 1999; Shinmura et al., 2000; Gu and Lu, 2001
	A:G	A	Slupska et al., 1999; Shinmura et al., 2000; Gu and Lu, 2001
	2-OH-A:G	2-OH-A	Ushijima et al., 2005

2-hydroxyadenine (2-OH-A) is a lesion that is induced by Fenton-type ROS and is produced for instance by H_2O_2 treatment of cultured mammalian cells (Jaruga and Dizdaroglu, 1996). Incorporation of 2-OH-dATP into the bacterial genome by pol III was shown to yield slightly increased mutant frequencies in a MutY deficient background in *E. coli*, suggesting that the processing of 2-OH-A damage possibly also involves the action of MutY (Kamiya and Kasai, 2000a). However, follow-up work by the same authors showed that, irrespectively of the base in the complementary strand, DNA with 2-OH-A presented a very poor substrate for MutY, and therefore illustrated that neither MutY nor Fpg seemed to play a role in 2-OH-A removal from DNA (Kamiya and Kasai, 2000b). Another result by Hashiguchi et al. again reassessed this finding and they reported that MutY indeed bound to 2-OH-A in duplex with G, A, or C and displayed a DNA glycosylase activity capable of removing 2-OH-A from 2-OH-A:G mismatches, which was dependent on the C-terminal domain of the protein (Hashiguchi et al., 2002).

FapyG is a DNA lesion that arises from oxidative stress by ring-fragmentation of the purine base. MutY excised A from A:FapyG mismatches, and this reaction was faster than the removal of A from A:G, but still slower than that from A:8-oxo-G *in vitro* (Wiederholt et al., 2003).

One group reported that MutY efficiently recognized 7-deaza-2′-deoxyadenosine (Z) and its non-polar isostere 4-methylindole-beta-deoxynucleoside (M) opposite 8-oxo-G and G in DNA, with a preference for M:8-oxo-G over Z:8-oxo-G mispairs (Chepanoske et al., 2000b). This finding was contradicting a previous report, in which Z:G mispairs were neither bound nor processed by MutY (Lu et al., 1995).

Lu et al. showed that MutY competes with and inhibits endonuclease VIII on its natural substrate, the hydroxyurea (hoU):A mismatch (Lu et al., 2006b).

A MutY variant from *Thermus thermophilus* processed A:8-oxo-G, G:8-oxo-G as well as T:8-oxo-G and A:G mismatches, but in contrast to other MutY variants, was shown to harbor a bifunctional glycosylase activity (Back et al., 2006).

MutY—enzymatic activity

The cloning of *E. coli* MutY revealed that it shared significant sequence homology to the bacterial endonuclease III (EndoIII), which acts on damaged base pairs (Michaels et al., 1990). MutY was shown to be an iron-sulfur (Fe-S) cluster protein containing both N-glycosylase and a 3′ AP endonuclease activity (Tsai-Wu et al., 1992). Initially there was some confusion regarding the enzymatic activity of MutY. While some reports stated that MutY also acted as an endonuclease on AP sites, therefore functioning as a bifunctional glycosylase (Tsai-Wu et al., 1992; Lu et al., 1995, 1996; Gogos et al., 1996; Manuel and Lloyd, 1997), Zharkov and Grollman showed that MutY does not harbor any AP lyase activity (Zharkov and Grollman, 1998). They hypothesized that the previous observations for the observed AP-activity were rather caused by heat-induced cleavage of the AP site and not due to an actual enzymatic activity. Moreover, this report suggested that the tight binding of MutY to its DNA substrate prevented the access of another bacterial glycosylase, the formamidopyrimidine-DNA glycosylase (Fpg), to the substrate. Consequently, MutY seemed to prevent a possible generation of a DNA double-strand break (DSB) by Fpg and thus possibly to play a role in the regulation of BER.

MutY—catalytic mechanism

When considering the catalytic activity of MutY (or any other DNA glycosylase), it is important to keep in mind that the catalytic cycle can be roughly subdivided into different stages, namely (1) recognition and binding of the enzyme to the substrate, (2) hydrolysis of the N-glycosidic bond or base-excision, and (3) dissociation of the enzyme or release of the resulting AP site. We have tried to structure the discussion according to these three steps in the catalytic cycle, whenever possible.

Substrate recognition. Multiple studies elucidating the contributions of the different parts of the MutY protein have been undertaken. Proteolytic digestion of MutY with thermolysin produced two fragments, an N-terminal one of 25 kDa and a C-terminal one of 12 kDa, respectively (Gogos et al., 1996). While the 12 kDa fragment did not display any detectable enzymatic activity, it was found to play an important role in the repair of mismatched oxidized DNA, as its deletion significantly impaired the binding and activity of MutY on A:8-oxo-G substrates, while it did not influence binding and cleavage of A:G substrates. On the other hand, a similar study, generating a 26 kDa N-terminal domain of MutY by trypsin-mediated proteolysis showed that this 26 kDa subunit was catalytically active, contained both DNA glycosylase and AP lyase activity, and was functionally identical with the full-length protein (Manuel et al., 1996; Manuel and Lloyd, 1997). A 14 kDa C-terminal domain of MutY (AA 1–226) was demonstrated to be the principal determinant for 8-oxo-G specificity, as its deletion remarkably enhanced the dissociation of the enzyme from A:8-oxo-G and reduced the rate of A removal from these substrates compared to A:G mismatches (Noll et al., 1999). This was interpreted such that the C-terminal domain facilitated A base flipping. Also, this study found that the C-terminal domain of MutY showed homology with MutT, suggesting that it might serve in 8-oxo-G recognition. Another report supported this view by showing that the N-terminal domain of MutY (AA 1–226) had a 18-fold lower affinity for binding various 8-oxo-G mismatches, a reduced catalytic preference for A:8-oxo-G over A:G mismatches and exhibited a lower inhibition on Fpg activity than the wild-type (wt) MutY (Li et al., 2000). These results suggested that the C-terminal domain of the protein determines its 8-oxo-G specificity and is crucial for mutation avoidance. The C-terminal domain was then shown to mediate additional contacts between MutY and A:8-oxo-G containing substrates that are not found in interaction with A:G (Li and Lu, 2000), thereby promoting the efficient recognition of substrates by MutY (Chmiel et al., 2001) and also affecting the catalytic activities toward A:G mismatches (Li and Lu, 2003). Taken together, the C-terminal domain of MutY seems to contribute substantially to the A:8-oxo-G substrate recognition.

It is still not entirely clear, how MutY is capable to efficiently recognize all its substrates from among the vast amount of undamaged base pairs. Along this line, the Fe-S cluster present in MutY was shown to be critical for the specific recognition of

its DNA substrate and its enzymatic activity (Porello et al., 1998a; Golinelli et al., 1999; Chepanoske et al., 2000a). It has also been suggested that the relative oxidation resistance of the Fe-S cluster may be an important aspect to guarantee the activity of MutY under conditions of oxidative stress (Messick et al., 2002). K142 in MutY, earlier shown to be involved in formation of tight interactions with DNA, was shown to make specific contacts with 8-oxo-G, and DNA-mediated charge transport (CT) was suggested as signal to promote the binding of MutY to DNA from a distance (Boon et al., 2002). Along this line, DNA-mediated CT led to oxidation of DNA-bound MutY, suggesting that G radicals provide the signal to stimulate DNA repair by the redox activation of DNA repair proteins through CT (Yavin et al., 2005). Further substantiating this idea, Boal et al. proposed that the rapid redistribution of proteins to the sites of DNA damage was mediated through redox activation involving the Fe-S clusters in proteins such as MutY and EndoIII (Boal et al., 2005; Yavin et al., 2006). A theoretical study of the DNA damage recognition by *Bacillus stearothermophilus* MutY proposed that the CT from MutY to DNA through hole transfer, which is specially efficient near an 8-oxo-G, leads to the stabilization of the enzyme in a conformation required for recognition of the lesion (Lin et al., 2008). Examination of the charge-transfer model by atomic force microscopy further validated this concept and emphasized the possibility that indeed repair proteins might be recruited to DNA lesions by DNA-mediated CT in the cellular context (Boal et al., 2009). The authors therefore proposed a model wherein the binding of Fe-S cluster containing DNA repair proteins (such as MutY and EndoIII) to DNA activates them toward oxidation. First, the formation of a guanine radical oxidizes a repair protein bound to DNA and thus stabilizes the binding of this protein. This step is followed by the binding of a second protein near the first one. Because also this protein gets oxidized during binding and transfers an electron to the DNA, it will induce a DNA-mediated CT from the second to the first protein if no damage is present in the DNA stretch between the two binding sites. This CT leads to reduction of the first protein and thus to its release from DNA, because in the reduced state it has a lower affinity to DNA. However, if there is a DNA lesion between the two bound proteins, the CT does not take place (it is "blocked" by the intervening lesion). In this situation both of the proteins remain bound and can subsequently catalyze repair steps. Through examination of CT mutants of EndoIII the group subsequently linked the ability of a repair protein to carry out DNA CT and its ability to localize to damaged DNA and thus further underlined their model (Romano et al., 2011). Taken together, the role for the Fe-S cluster as redox cofactor to search for damaged bases using DNA-mediated CT becomes more and more substantiated and really presents a plausible scenario to explain the mechanisms of full-genome search for lesions.

Base-excision. Investigations into the glycosylase activity of MutY revealed a distinctive difference in the processing of A:8-oxo-G compared to A:G mismatches (Porello et al., 1998b). Hydrolysis of A from opposite 8-oxo-G was at least 6-fold faster than from the A:G mispair. Interestingly however, MutY "lingered" when excising from an A:8-oxo-G base pair and released the product with a much slower kinetic compared to the A:G substrate. This delay in substrate release might protect 8-oxo-G from being prematurely accessed and removed by other glycosylases, as also suggested by Zharkov and Grollman (1998). A detailed study of the active site revealed the importance of several amino acids involved in the glycosylase as well as DNA binding activities of MutY (Wright et al., 1999). Bifunctional glycosylases all bear a conserved lysine residue believed to be important for the initial nucleophilic attack in base removal near their active site, which is lacking in their monofunctional counterparts. To yield more insight into the role of this residue on a structural basis, Williams et al. investigated whether insertion of such a lysine residue into the catalytic site of MutY had any influence on its activity. Indeed, a point-mutation at S120K generated a MutY mutant capable of catalyzing DNA strand scission at a rate that was similar to its A excision activity from A:G and A:8-oxo-G substrates, and also changed it into a bifunctional glycosylase (Williams and David, 2000). This study illustrated that the basic mechanisms of mono- and bifunctional glycosylases were quite similar. The glycosylase activity of MutY was shown to involve a Schiff base intermediate, characteristic for other bifunctional DNA glycosylases that catalyze a β-lyase reaction, though no β-lyase step (*per se* only performed by bifunctional glycosylases) could be observed (Williams and David, 1998). Reduction of this Schiff-base intermediate with borohydride resulted in the formation of a covalent MutY-DNA adduct. To identify the residues involved in this covalent complex formation, Williams et al. constructed different MutY mutants and identified K142 to be the primary residue for such covalent associations (Williams and David, 1999). As the DNA binding and enzymatic activity of the K142A mutant was comparable to that of the wt enzyme, the formation of this covalent intermediate was not required for removal of A and was suggested to be a consequence of the unusually high affinity of MutY for the product of its glycosylase activity. Similarly, mutation of K142 to glutamine in MutY was shown to also abrogate its ability to form a Schiff base with DNA, while still retaining some of its catalytic activity (Zharkov et al., 2000). Interestingly, this mutation selectively impaired the processing of A:G base pairs, but not of A:8-oxo-G substrates, primarily by interfering with the binding to A:G substrates, but did not impair the catalytic activity *per se*, again confirming that it was not directly involved in the catalytic step. Using unnatural substrates to elucidate the tolerance of MutY to different modifications of the A or the 8-oxo-G in mismatches in an *E. coli*-based cellular assay, it was seen that, while modification of A was tolerated rather well, modification of 8-oxo-G resulted in a drastic reduction of base-excision (Livingston et al., 2008). This led to the conclusion that the presence of 8-oxo-G is critical for MutY to recognize A:8-oxo-G mismatches *in vivo* to initiate repair. D138 and Q37 are both residues that are involved in the catalytic mechanism of MutY-mediated A removal. Interestingly, their substitution yielded mutants with a range of different excision activities. Studies of these mutants demonstrated that changes which reduced the excision activity were better tolerated and less compromising to A:8-oxo-G repair *in vivo* in *E. coli* than those affecting the recognition of A:8-oxo-G mismatch affinity (Brinkmeyer et al., 2012). Therefore, this report suggested that the recognition of A:8-oxo-G mismatches was more important for the

correct repair of these duplexes than the actual glycosylase activity *per se*. Interestingly, this can be reconciled with the fact that the release of the substrate by MutY after base-excision is much slower than the actual N-glycosidic activity, seemingly demonstrating that the rate-limiting step of this enzyme is rather the identification of its substrate than the excision step itself. Additionally, this study also revealed which residues are critical for the selectivity and specificity of MutY.

Substrate release. The product release rate of MutY could be greatly enhanced by the two proteins AP-endonuclease IV and exonuclease III, and this effect depended on the presence of the C-terminal domain of MutY (Pope et al., 2002). Also, endonuclease VIII was found to promote MutY dissociation from AP:G substrates, but not from AP:8-oxo-G, and to further process these by βδ elimination (Lu et al., 2006b). This study also showed that MutY interacts with endo VIII through its C-terminus and competes with endo VIII on its natural substrate, the hydroxyurea (hoU):A mismatch, thus inhibiting its activity and possibly reducing the mutagenic effects of hoU. Taken together, it seems important that also the substrate release step is tightly regulated, in order to orchestrate the following steps and to protect the 1-nt gap resulting from base-excision.

Structure of MutY and the removal of adenine opposite 8-oxo-G

The most precise structure of MutY comes from studies with *Bacillus stearothemophilus* (Lee and Verdine, 2009) (**Figure 2**). After binding to the 8-oxo-G:A mispair MutY flips out the A from the DNA double-helix. A water molecule is positioned between Asp144 and Asn146 in the MutY lesion-recognition pocket of the enzyme. Earlier studies included biochemical and computational studies on uracil DNA glycosylase (Werner and Stivers, 2000; Dinner et al., 2001) suggested that a so called dissociative action occurs, where the cleavage of the N-glycosylic bond and the subsequent attack of the water molecule on the C1′ (arrow in **Figure 2A**) do not occur simultaneously, but rather in two

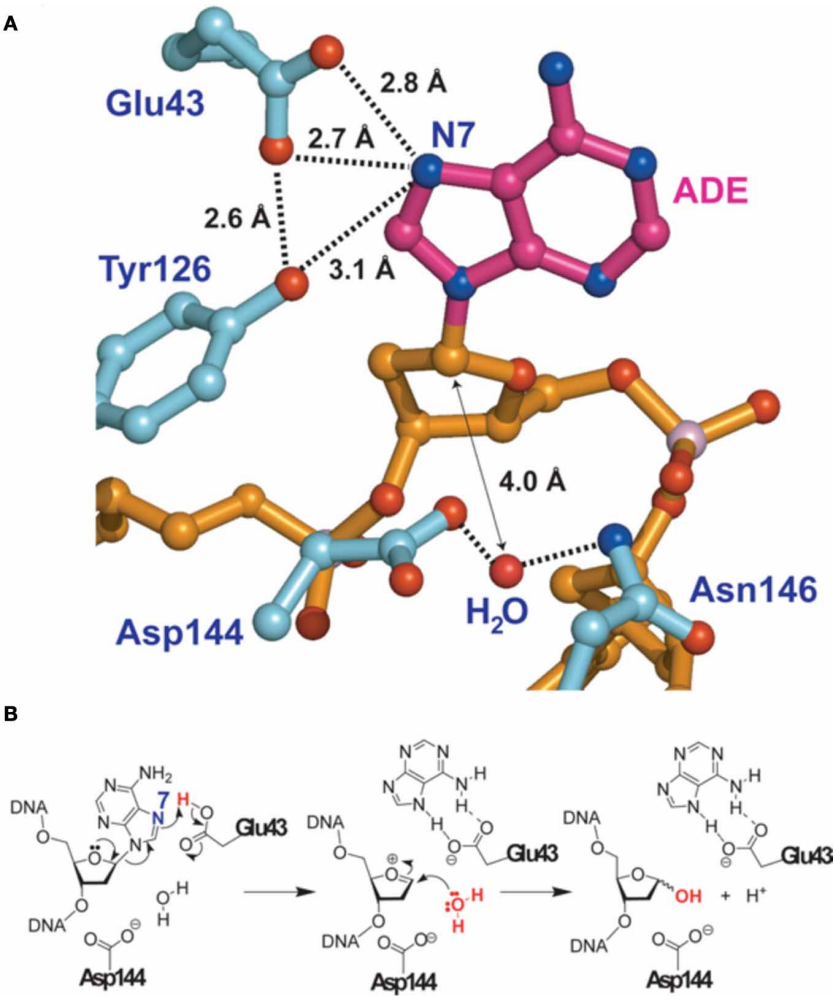

FIGURE 2 | Adenine removal by MUTYH. (A) View of the substrate adenosine interacting with catalytic residues of MUTYH. **(B)** Proposed glycolytic mechanism based on the structural information of **(A)**. Reproduced form Lee and Verdine (2009). For details see text.

discrete steps. In addition Glu43 can adopt a so-called bifurcated hydrogen-bonding interaction of 2.7 and 2.8 Angströms, respectively, with N7 of A (**Figures 2A,B**). These short distances together with a protonated Glu43, provides acidity and therefore full hydrogen bonding to the N7 of A. As indicated in **Figure 2B** such a conformation favors the scission of the glycosylic bond between A and the deoxyribose. A similar structure has also been identified for human MUTYH, for which a fragment lacking the first 64 amino-acids was crystalized (Luncsford et al., 2010).

The structure of MutY catalytic core revealed that the two helical domains form a positively-charged groove, positioning the A-binding pocket at their interface (Guan et al., 1998). Also, this study confirmed a nucleotide flipping mechanism by a substitution of the Watson–Crick hydrogen bond partners by protein atoms. Recognition of 8-oxo-G seems to occur independently of double-stranded DNA or of an A-mismatch, and sequential extrusion of 8-oxo-G followed by A occurs in MutY, as demonstrated by Bernards et al. (2002). MutY has been proposed to assemble into a dimer upon substrate binding to yield an active form of the enzyme (Wong et al., 2003). This idea was further substantiated by a study that suggested a model for MutY binding of the mismatched DNA that involves scanning of the DNA by one molecule which enhances binding of second MutY molecule upon encountering an A:8-oxo-G mismatch (Lee et al., 2004).

Kinetically, it has been suggested that the release of A happens fast, while the rate-limiting step was the release of the AP-site (McCann and Berti, 2003). Further investigations into the transition state structure of MutY showed that the irreversible breakage of the N-glycosidic bond could not take place until a H_2O atom was present and that the enzyme stabilized the excision site after excision (McCann and Berti, 2008). Recently, a two-step reaction was proposed to be the basis of the catalytic activity of MutY, as opposed to the three-step mechanism proposed before (Tiwari et al., 2011).

Investigations of the roles of the different H_2O molecules involved in catalysis by MutY from *B. stearothermophilus* and *E. coli* suggested that E43 and N7 may be important factors for the activity of MutY (Brunk et al., 2012). Further insight into the roles of the substrate A residues N7 and N3 during catalytic excision by MutY have been gained recently (Michelson et al., 2012).

MutY in living cells

In *E. coli*, MutY was shown to be co-transcribed as first gene of a part of a large operon, together with Fpg, the bacterial DNA glycosylase which removes 8-oxo-G from the DNA (Gifford and Wallace, 1999). This further emphasized the involvement of MutY in the repair of 8-oxo-G base pairs in an interplay with Fpg and thus in the response to oxidative DNA damage. Somewhat surprisingly at first glance, oxidative stress down-regulated the activity of MutY by 70% as well as its mRNA levels, and in contrary it was induced more than 2-fold under anaerobic conditions (Yoon et al., 2003). This negative regulation of MutY was mediated by the regulatory genes fur, fnr and arcA. These results were explained with the idea that MutY activity had to be restrained when increased incorporation of 8-oxo-dGTP could possibly take place, which is during times of oxidative stress. This is important, because 8-oxo-dGTP could be inserted opposite a correct templating A, which might erroneously get excised by the action of MutY, thus actually acting *promutagenic* in this scenario instead of protecting from mutations taking place.

Screening for mutator loci leading to GC→CG transversions in *E. coli*, Zhang et al. found that inactivation of MutY led to accumulation of these mutations (Zhang et al., 1998). As mentioned above, they showed that MutY bound to G:8-oxo-G mismatches and was capable of removing G from the G:8-oxo-G mispair.

To analyze the impact of *mutT, mutM* (which encodes the Fpg DNA glycosylase that removes 8-oxo-G from C:8-oxo-G base pairs in bacteria), and *mutY* on the mutational spectra, following considerations have to be taken into account. In the context of 8-oxo-G and 8-oxo-dGTP (1) CG→AT mutations can arise either from oxidation of C:G to C:8-oxo-G or from incorporation of 8-oxo-dGTP opposite C, followed by wrong incorporation of A opposite 8-oxo-G by the replicative pols during the next round of replication. (2) AT→CG mutations are based on incorporation of 8-oxo-dGTP opposite templating A. Analyzing different combinations of mutated strains in *mutT, mutY,* and *mutM*, Fowler et al. found that (1) *mutT* does not increase CG→AT transversions, regardless of the *mutY* and *mutM* background, suggesting that 8-oxo-dGTP does not often get incorporated opposite C but rather opposite A. (2) AT→CG transversions are reduced in *mutY* and *mutMmutY* backgrounds, suggesting templating 8-oxo-G preferentially pairs with dATP, which then is a substrate for MutY to excise A from the A:8-oxo-G pair, followed by Fpg that removes 8-oxo-G paired with C. And finally (3) *mutY* and *mutMmutY* decrease AT→CG mutations (arising from incorporation of 8-oxo-dGTP opposite templating A) in a *mutT wt* background, suggesting that a certain amount of 8-oxo-G gets incorporated into DNA even in the presence of functional MutT (Fowler et al., 2003). No strand bias in the mutation rate between leading and lagging strand synthesis in either a *mutMmutY* or a *mutT* background could be detected in *E. coli* using a supF shuttle vector (Watanabe et al., 2001). Interestingly, Bridges et al. showed that the rate of mutation markedly increased in starved *mutY* mutant *E. coli*, yielding CG→AT transversion mutations (Bridges et al., 1996). This phenotype could be further enhanced by additional mutation of *mutM*, even though mutation of *mutM* alone did not cause this effect. Also, addition of catalase to the plates did not alter the mutation rates, indicating that extracellular H_2O_2 was not involved in the generation of mutations, and it was suggested that singlet oxygen could be the source of internal DNA damage. These findings indicated that MutY may regulate the activity of Fpg in resting cells. Expression of MutY from a *mutY-lacZ* fusion construct was shown to be enhanced under aerobic compared to anaerobic conditions, but not to be down-regulated by nutrient limitation (Notley-McRobb et al., 2002). However, in many cases, nutrient limitation led to *mutY* inactivation by deletion, suggesting it might serve as a mechanism to increase mutation rates under these adverse conditions.

Clustered lesions, as induced by ionizing radiation, are defined as two or more lesions formed within one to two helical turns of the DNA. They present a challenge to the repair machinery of the cell. An 8-oxo-G in the vicinity of an AP site was found to retard the processing of the AP site by endo III and Fpg, and the AP site was found to elevate the mutation frequency at

8-oxo-G in *wt, nth, fpg,* and *mutY* deleted *E. coli* (Cunniffe et al., 2007). Interestingly though, the mutation frequency in *mutYfpg* null cells was reduced by the presence of the AP site, suggesting that the processing of tandem lesions challenges the cellular repair machineries. Similar findings by Noguchi et al. investigating the interplay of 1-nt-gaps and 8-oxo-G lesions in clusters in *E. coli* demonstrated again, that the mutagenic potential of 8-oxo-G depends on the presence and the position of the gap (Noguchi et al., 2012).

MutY competed with MutS-dependent mismatch-repair when A:C mispairs were present, especially in the presence of an increased dCTP pool (Kim et al., 2003). In *E. coli,* MutY has been shown to interact via its Fe-S cluster with the ATPase domain of MutS, which enhanced the binding affinity of MutY to A:8-oxo-G mismatches (Bai and Lu, 2007). MutY expression and activity were enhanced in a *mutS* mutant strain, and AT→GC transversions were reduced by additional mutation of mutY in a mutS background, suggesting a cooperative effect of MutY and MutS in repair of 8-oxo-G damage. Analysis of *Bacillus subtilis* revealed that the expression of MutY increased drastically upon deletion of *mutSL* operon in starved cells, possibly to disturb the balance between MutY and MMR proteins to support the production of mutations, which might give growth advantages to these cells (Debora et al., 2011).

In *Streptococcus mutans*, an oral pathogen, strains with mutations of *mutY* were shown to display elevated mutation rates, increased resistance to killing by acid and oxidative agents as well as higher virulence compared to the parent strain, suggesting that loss of a BER factor such as MutY could confer an advantage to pathogenic organisms (Gonzalez et al., 2012).

MutY and BER in E. coli

Reconstitution experiments with purified proteins from *E. coli* revealed, that the presence of Ape1, pol I, and DNA ligase is sufficient to catalyze the entire repair pathway of G:A mismatches *in vitro* (Au et al., 1989). Further elucidation of the pathway was achieved, when Radicella et al. showed that the average repair tract length initiated by MutY in *E. coli* is 9–27 nucleotides long, starting at the removed A, and involved pol I, even though the involvement of other pols was also evident (Radicella et al., 1993). This finding was further refined *in vitro*, when Tsai-Wu et al. found pol I to be responsible for generating these tracts of 5–12 nucleotides length (Tsai-Wu and Lu, 1994).

MUTYH

MUTYH activity and substrate specificity

The substrate specificities for MUTYH are summarized in **Table 1**. The mammalian homolog of MutY, MUTYH, was first purified from calf thymus and catalyzed removal of A from A:G, A:8-oxo-G and A:C mismatches (McGoldrick et al., 1995). Subsequently, expression and purification of the cloned human protein confirmed its activity to remove A from A:8-oxo-G and A:G base pairs *in vitro*, supporting that also the human homolog is a *bona fide* DNA glycosylase (Slupska et al., 1999). Purification of MUTYH from calf liver mitochondria yielded a protein that complexes with A:8-oxo-G, G:8-oxo-G, and T:8-oxo-G, weakly with C:8-oxo-G but not with A:G and A:C mismatches and removed A mispaired with G, C, or 8-oxo-G while weakly removing G from G:8-oxo-G mispairs (Parker et al., 2000). Purification of the murine MUTYH revealed strong similarities to MutY function, even though the intrinsic rates of A removal were lower than by MutY (Pope and David, 2005). Shinmura and colleagues reported that both the purified nuclear and mitochondrial recombinant isoforms of human MUTYH were active, and predominantly removed A from A:8-oxo-G mispairs rather than A:G mispairs under physiological salt concentrations (Shinmura et al., 2000). MUTYH in human cell extracts was shown to be more active in binding and glycosylase activity toward A:G mismatches than recombinant MUTYH expressed in bacteria (Gu and Lu, 2001). Furthermore, the authors found this native form of MUTYH to migrate slower on a non-denaturing polyacrylamide gel than recombinant human MUTYH purified from bacteria. Moreover the native form seems to be phosphorylated, thus apparently enhancing its glycosylase activity predominantly on A:G but also on A:8-oxo-G. As the phosphorylation status of MUTYH did not alter its electric mobility, it was suggested to be possibly associated with other proteins to account for the higher apparent molecular weight. Accordingly, co-migration of APE1 and MUTYH with A:8-oxo-G substrates could be identified. Ohtsubo et al. found that MUTYH likely also harbors an activity to remove 2-OH-A (Ohtsubo et al., 2000). Removal of 2-OH-A from opposite 8-oxo-G or G has been described for murine MUTYH (Pope and David, 2005) and was confirmed for human MUTYH as well (Ushijima et al., 2005). MUTYH from *S. pombe* was able to remove G from G:8-oxo-G mismatches as efficiently as A from A:8-oxo-G mismatches, and its expression reduced the frequency of GC→CG transversions in an *E. coli mutY* mutant, suggesting it might be involved in the repair of G:8-oxo-G lesions (Doi et al., 2005).

A:8-oxo-G substrates processed by murine MUTYH were protected from inappropriate access by OGG1 and APE1, thus preventing the formation of DSBs (Tominaga et al., 2004).

A study by Miyako et al. found that mitochondrial DNA (mtDNA) from HeLa cells could be cleaved by recombinant *E. coli* MutY, in contrast to Fpg which has been shown to barely cleave mtDNA (Driggers et al., 1993; Hegler et al., 1993), and that this cleavage took place roughly at a rate that was expected to correspond to the amount of 8-oxo-G present in endogenous mtDNA (Miyako et al., 2000). Suzuki et al investigated the repair of 8-oxo-G in DNA and 8-oxo-dGTP in 293T cells using supF shuttle plasmids (Suzuki et al., 2010). While knockdown of OGG1, MUTYH, NTH1, and NEIL1 all led to a significant increase in CG→AT transversions caused by the C:8-oxo-G pair in the shuttle plasmid, only knockdown of MUTYH resulted in a reduction in AT→CG transversions induced by 8-oxo-dGTP. In summary, MUTYH displays remarkable similarity to its bacterial homolog MutY regarding its activity and substrate specificity.

Localization of MUTYH

The subcellular localization of MUTYH was rather enigmatic for a long time. A study using expression of tagged proteins in COS-7 cells revealed that MUTYH was mainly transported to the mitochondria, which was probably the result of the isoform that was used (Takao et al., 1998). Follow-up work by the same group

identified an alternatively spliced transcript differing in exon 1, leading to the nuclear localization of this variant (Takao et al., 1999). Ten further isoforms containing unique 5′sequences that could be grouped into three types were subsequently described, and suggested to encode multiple authentic MUTYH proteins (Ohtsubo et al., 2000). Other reports have further discussed the localization of MUTYH in cells, finding isoforms targeted to the nucleus (Tsai-Wu et al., 2000; Ichinoe et al., 2004) or the mitochondria (Englander et al., 2002; Ichinoe et al., 2004). However, work still needs to be done to analyze the distribution of isoforms to the different subcellular compartments in different cell and tissue types to clarify this matter further.

Analyzing the distribution of endogenous MUTYH in serum-stimulated proliferating MRC5 cells with antibodies, Boldogh et al. reported both nuclear and mitochondrial localization of MUTYH (Boldogh et al., 2001). The nuclear form co-localized with BrdU and the proliferating cell nuclear antigen (PCNA) and, similarly to PCNA, increased 3- to 4-fold to peak during S-phase compared to G1, whereas levels of OGG1 or MTH1 did not change during the cell cycle. These studies suggested a role of targeting MUTYH to the replication fork to ensure that its activity is directed to the newly synthesized template strand. Subsequently, DNA replication was shown to enhance the MUTYH-dependent repair of A:8-oxo-G mismatches *in vivo*, and it was demonstrated that the interaction with PCNA was critical for the activity of MUTYH (Hayashi et al., 2002). Taken together, these findings clearly support a replication-associated function of MUTYH.

MUTYH and DNA damage signaling

Recently, a number of reports have accumulated that link MUTYH to the DNA damage response and implicate it in apoptotic signaling. To investigate the contribution of nuclear and mitochondrial accumulation of oxidative base lesions to the triggering of apoptosis, Oka and colleagues used *OGG1* knockout (ko) cells deficient in the nuclear or mitochondrial form of MUTYH, respectively (Oka et al., 2008). The accumulation of single-strand breaks in nuclear DNA triggered PARP-dependent cell death and could be rescued by depletion of nuclear MUTYH. The same was true for mitochondria, where MUTYH triggered calpain-dependent cell death by single-strand breaks. These results suggested that MUTYH catalyzes the formation of single-strand breaks in both of these DNAs, hence leading to the execution of apoptosis. Exposure of human cells to sodium nitroprusside, an agent that causes 8-oxo-G accumulation in cellular DNA by acting as an NO donor, led to MUTYH-dependent cell death that was initiated by oxidized bases in the mitochondrial, but not the nuclear DNA (Ichikawa et al., 2008). The role of single-strand breaks generated by MUTYH in the induction of cell death was further underlined by the finding that synthetic sickness/lethality mediated by either inhibition of pol β combined with MSH2, a component of the mismatch repair pathway, or pol γ with MLH1, both of which led to a nuclear 8-oxo-G accumulation, could be rescued by silencing of MUTYH (Martin et al., 2010). BER has been implicated in many different pathological conditions of the central nervous system (reviewed in Bosshard et al., 2012). A very recent report implicated MUTYH in degeneration by triggering apoptosis in microglia and neurons through initiation of single-strand breaks during repair of A:8-oxo-G mismatches (Sheng et al., 2012). Nuclear accumulation of 8-oxo-G triggered PARP-dependent apoptosis in microglia, while mitochondrial 8-oxo-G accumulation led to calpain-dependent apoptosis in neurons. All these findings are in agreement with a model, wherein the repair of DNA mismatches by MUTYH leads to generation of toxic single-strand breaks, and thus contributes to cellular death in case of excessive damage burden (i.e., an amount of DNA damage that surpasses the cellular capacity to further process these lesions properly). Thus, this model explains, why under conditions of severe damage the *absence* of MUTYH is *beneficial for the survival* of the cells. On the other hand, there are a number of reports that show that *loss* of MUTYH actually can *sensitize* cells to DNA damaging agents. Along this line, double mutations in *S. pombe MUTYH* with *RAD1* or, to a lesser extent *RAD9*, were shown to enhance the sensitivity of the cells to DNA damaging agents and hydroxyurea (Jansson et al., 2008). The consequences of these deficiencies were chromosome segregation defects and checkpoint failure after UV irradiation, as well as morphological defects, even in the absence of DNA damaging agents. This implicated MUTYH in the repair of a wide range of DNA damage and linked it to the checkpoint pathway. Under low-dose oxidative stress, *MUTYH OGG1* double-ko mouse cells also showed hypersensitivity to oxidation damage and a reduction of S phase concomitant with an increase of G2/M phase cells, while the levels of cell death remained unchanged (Xie et al., 2008). Furthermore, an increase in centrosome amplifications and formation of multinucleated cells could be observed in the surviving fraction of the ko cells, suggesting an involvement of MUTYH and OGG1 in the regulation of cell-cycle progression and cell division under oxidative stress. Further evidence implicating MUTYH in checkpoint control came from a study showing that siRNA-mediated knockdown of MUTYH resulted in a decreased phosphorylation of ATR and Chk1 upon treatment of HEK293 cells with HU or UV (Hahm et al., 2011). Concomitantly, the authors observed an increase in the phosphorylation of Cdk2 as well as the amount of the Cdc25A phosphatase, suggesting that MUTYH was involved in activation of the DNA damage response.

Thus, there seems to be growing evidence that implicates MUTYH to be an important factor in the cellular response to oxidative stress and inflammatory conditions by an involvement in cell death signaling (as discussed in Oka and Nakabeppu, 2011). Along these lines, MUTYH has been suggested to play a role in mitochondrial dysfunction in the pathogenesis of Parkinson's disease (Fukae et al., 2007; Nakabeppu et al., 2007). However, it still remains to be clarified how MUTYH can initiate apoptosis of cells in some instances, while it seems to protect from apoptosis in others.

Impact of MUTYH knockout on oxidative DNA damage and tumorigenesis in vivo

The data on cells and mice with biallelic deletion of MUTYH are somewhat discrepant. MUTYH ko embryonic stem cells displayed a mutator phenotype, but did not show any hypersensitivity toward oxidative stress induced by H_2O_2 or menadione (Hirano et al., 2003). A study with *mutyh*$^{-/-}$ knockout mice by Xie et al revealed no significant increase in survival or tumor incidence

after 14 months, suggesting that MUTYH deficiency is not sufficient to cause a tumor-predisposition (Xie et al., 2004). This study also showed that combined ko of MUTYH with OGG1 led to a decrease in life span and increased tumor formation for double ko mice compared to single ko. Interestingly, 75% of the lung tumors showed an activating GC→TA transversion mutation at codon 12 of K-ras, a feature that is often detected in MUTYH-associated polyposis (MAP) tumors, but none in the p53 gene or in the adjacent normal tissues. Additional heterozygosity for Msh2 (*mutyh*$^{-/-}$ *ogg1*$^{-/-}$ *msh2*$^{\pm}$) did not inflict on the total tumor incidence but accelerated malignant lung and ovarian tumor formation in the *mutyh*$^{-/-}$ *ogg1*$^{-/-}$ background. A complete knockout of Msh2 to generate triple ko (*mutyh*$^{-/-}$ *ogg1*$^{-/-}$ *msh2*$^{-/-}$) further increased tumor incidence and decreased survival time, but did not differ from the phenotype displayed by *msh2*$^{-/-}$ single knockouts. This was suggested to be due to the strong mutator phenotype of *msh2*$^{-/-}$ mice that might mask additional difference caused by *mutyh*$^{-/-}$ and *ogg1*$^{-/-}$.

Spontaneous mutagenesis in the small intestine of *ogg1*$^{-/-}$ and *mutyh*$^{-/-}$ *ogg1*$^{-/-}$ double deficient mice at the age of 4–5 weeks using a transgene reporter revealed increased mutations in the double-ko's but not in the *ogg1*$^{-/-}$ mice (Isogawa, 2004). Furthermore, the GC→TA mutation frequency increased in *mutyh*$^{-/-}$ and in *ogg1*$^{-/-}$and a cooperative increase could be observed in *mutyh*$^{-/-}$ *ogg1*$^{-/-}$, suggesting a cooperative function between OGG1 and MUTYH to prevent 8-oxoG-related mutagenesis. Russo et al. also reported an additive effect in *mutyh*$^{-/-}$ *ogg1*$^{-/-}$ on the age-dependent increase in 8-oxo-G levels in lung and small intestine compared to the single ko's (Russo et al., 2004). Strikingly, these tissues were identical with the ones that showed increased cancer incidence in *mutyh*$^{-/-}$ *ogg1*$^{-/-}$ mice in the study by Xie et al. (2004). MUTYH deficiency in a background of APC$^{min/+}$ mice led to the occurrence of stop-codons in the APC gene by induction of CG→AT transversion mutations and thus promoted intestinal tumorigenesis (Sieber et al., 2004).

In 2007 a study reported an increased susceptibility to spontaneous and stress-induced tumorigenesis in a large cohort of *mutyh*$^{-/-}$ mice kept for 18 months, strongly contradicting data on *mutyh*$^{-/-}$ obtained by different groups thus far (Sakamoto et al., 2007). This suggested that presence of a MUTYH deficiency is sufficient to predispose for malignancies of the intestinal tract, such as lymphoma and adenoma. More impressively still, oral $KBrO_3$ treatment of *mutyh*$^{-/-}$ mice led to a dramatic increase in CG→AT transversion mutations and small intestinal tumors. The authors claimed that the tumor-prone phenotype might have been missed earlier due to genetic differences in the mouse strains and the older age at which the tumor burden was evaluated in their study. This was in line with the fact that many of the studies with *mutyh*$^{-/-}$ mice have been reporting a strong tendency toward age-dependent accumulation of 8-oxo-G in tissues. In general, in light of the huge complexity of the disease, it can be debated, whether mice are useful cancer models to compare with the human disease, due to the entirely different life span, metabolism, inbreeding status and many other aspects.

As noted above, the combination *mutyh*$^{-/-}$ and *msh2*$^{-/-}$ did not greatly affect the mutation rate. However, the loss of *mutyh*$^{-/-}$ combined with *msh2*$^{-/-}$ significantly increased the amount of oxidative DNA damage in several organs compared to *msh2*$^{-/-}$ mice, suggesting an independent contribution of both genes to genetic maintenance (Russo et al., 2009). Surprisingly, the development of metastasizing lymphoma and the time of death were significantly delayed in the *mutyh*$^{-/-}$*msh2*$^{-/-}$ mice compared to *msh2*$^{-/-}$, suggesting that the cancer-prone phenotype of the double knockouts depends substantially on the activity of MUTYH (Russo et al., 2009). The relationship of MUTYH and MMR is reviewed in more detail in Russo et al. (2007).

In a mouse model of ulcerative colitis MUTYH was shown to play a major role in propagating the inflammatory response that lead to the onset of chronic colitis (Casorelli et al., 2010). Taken together, all the data analyzing the function of MUTYH *in vivo* strongly supports the idea that MUTYH-mediated correction of A:8-oxo-G mispairs plays an important role in the maintenance of genetic integrity and protects cells from malignant transformation.

THE MUTYH/POL λ BASE-EXCISION REPAIR PATHWAY

By catalyzing the excision of the mispaired A from A:8-oxo-G base pairs, MUTYH paves the way for a subsequent repair that ultimately reconstitutes an undamaged C:G base pair. MUTYH-initiated repair has been shown to involve a replication-coupled long-patch BER (LP-BER) pathway (Matsumoto, 2001; Parker et al., 2001; Yang et al., 2001; Parlanti et al., 2002). Along this line, a SP-BER pathway initiated by MUTYH was shown to be futile, because it uniquely generated A:8-oxo-G base pairs instead of the correct C:8-oxo-G base pairs, indicating that canonical MUTYH-initiated BER must proceed by the LP-BER sub-pathway (Hashimoto et al., 2004). For a long time it was unclear, which pol was capable of faithfully inserting a correct C opposite 8-oxo-G, as most examined pols showed significant error-prone bypass of 8-oxo-G (Shibutani et al., 1991; Pinz et al., 1995; Efrati et al., 1999; Prakash et al., 2000; Einolf and Guengerich, 2001; Vaisman and Woodgate, 2001; Krahn et al., 2003; Hsu et al., 2004). In 2007, our laboratory proposed that pol λ, together with its cofactors PCNA and replication protein A (RPA), inserts 1200-fold more efficiently the correct C opposite 8-oxo-G than the incorrect A (Maga et al., 2007). Furthermore, experiments with extracts from mouse embryonic fibroblasts (MEFs) deficient for pol λ suggested an important role of pol λ in bypass of 8-oxo-G. The importance of PCNA and RPA to determine the pol selection at 8-oxo-G lesions was further analyzed in a follow-up study. The two proteins were found to act as molecular switches to activate pol λ-dependent correct 8-oxo-G bypass and to repress the more error-prone pol β-dependent bypass (Maga et al., 2008). Subsequently, we showed that the MUTYH-initiated error-free LP-BER pathway involves pol λ (Maga et al., 2008; Van Loon and Hubscher, 2009), as depicted in detail in **Figure 1**. Herein, the monofunctional MUTYH excises the promutagenic A from A:8-oxo-G base pairs. This is followed by incision of the phosphodiester backbone 5′ to the AP site by APE 1 that generates a 3′OH and a 5′dRP moiety, respectively. Thereafter, in the presence of RPA and PCNA, pol λ incorporates the correct C opposite 8-oxo-G and further elongates the primer by one more nucleotide (nt) downstream, thus generating a short 1-nt 5′ flap. This overhang

is processed by flap endonuclease 1 (Fen1), resulting in a product that can be ligated by DNA ligase I. The resulting C:8-oxo-G base pair is then substrate for the canonical OGG1-initiated SP-BER as discussed above.

MUTYH-INTERACTING PROTEINS

All DNA damage repair pathways have to be tightly coordinated to ensure proper repair and to avoid the generation of cytotoxic and mutagenic intermediates. Protein-protein interactions either regulate the repair by recruitment of proteins to sites of DNA damage or modulate the catalytic activity of already bound enzymes.

MUTYH is interacting with proteins associated with the BER pathway, DNA replication and cell cycle checkpoints (**Table 2**). The first interaction partner of MUTYH was the endonuclease Ape1 (Parker et al., 2001; Yang et al., 2001). Ape1 stimulates the glycosylase activity of MUTYH independently from its own activity; a catalytically dead mutant of Ape1 still enhanced the cleavage efficiency of MUTYH on damaged DNA templates (Yang et al., 2001). Thus, the stabilization of the MUTYH-DNA complex was sufficient to enhance the repair capacity. Additionally, MUTYH and Ape1 were both recruited into a complex with A:8-oxo-G containing DNA in HeLa cell extracts (Gu and Lu, 2001). The interaction between the two proteins was suggested to be important to prevent the release of cytotoxic AP sites (Luncsford et al., 2010). MUTYH was found to interact with pol λ, as discussed above (Van Loon and Hubscher, 2009). Furthermore, the interaction of MUTYH with pol λ was enhanced by phosphorylation of pol λ by Cdk2/cyclinA (Markkanen et al., 2012b,c).

Gu et al identified the mismatch repair protein MSH6 as further interaction partner of MUTYH, and MSH6 regulated MUTYH by stimulating its glycosylase activity and binding capacity to A:8-oxo-G containing DNA (Gu et al., 2002).

MUTYH interacts with PCNA and RPA under conditions of unperturbed DNA replication. It was suggested that, upon encountering DNA damage, MUTYH switches to interact with the heterotrimeric ring-like molecule Rad 9, Rad1, and Hus 1, called the 9-1-1 complex (Parker et al., 2001; Shi et al., 2006). Consistent with these findings, MUTYH co-localized with PCNA at replication foci in untreated cells (Boldogh et al., 2001). Also, replication was a prerequisite for MUTYH mediated repair to occur (Hayashi et al., 2002). The interaction site with PCNA was mapped to a conserved region within the MutY family, reflecting the importance of this interaction since PCNA directs MUTYH to the daughter strand where it excises a recently inserted mispaired A from A:8-oxo-G base pairs (Slupska et al., 1999). This directionality could also be the mechanism to make sure that MUTYH does not excise erroneously A from a base pair where 8-oxo-dGTP has been inserted opposite a templating (and thus correct) A. The interaction of MUTYH with PCNA and the structurally-related 9-1-1 complex was also confirmed in *S. pombe* (Parker et al., 2001; Chang and Lu, 2002, 2005; Shi et al., 2006; Luncsford et al., 2010). Interestingly, it was shown that even if the SpMUTYH does not have a perfect PCNA binding motif (Chang and Lu, 2005), cross-binding between the yeast and the human isoforms is possible and mutations within the PCNA binding domain impair the capability of MUTYH to repair A:8-oxo-G mismatches in yeast (Chang and Lu, 2002).

The 9-1-1 complex acts as a DNA damage sensor, blocks the cell cycle and simultaneously stimulates BER to allow repair to be completed before the DNA is replicated. The human MUTYH interacts with the h9-1-1 complex via binding to hRad1 and hHus1, but not to hRad9 (Shi et al., 2006). The glycosylase activity of MUTYH was stimulated by this interaction if 9-1-1 was present in a substantial molar excess. Treatment of cells with H_2O_2 or ionizing irradiation enhanced this interaction, supporting the hypothesis that 9-1-1 replaces PCNA in stress situations (Shi et al., 2006). Luncsford et al. identified the interdomain connector (IDC) of MUTYH to mediate the binding to 9-1-1 by providing a stabilized docking interface and proved the importance of the interaction by showing that mutations within this site decrease the repair of oxidative damage *in vivo* (Luncsford et al., 2010).

Partial interchangeability was observed between human and *S. pombe* homologs of these proteins, and enhanced glycosylase

Table 2 | Interaction partners of MUTYH.

Interaction partner	Species	Interaction site in MUTYH	Stimulatory effect
Ape1	*human*	259–318 (Parker et al., 2001)	Glycosylase activity (Yang et al., 2001)
MSH6	*human*	232–254 (Gu et al., 2002)	Glycosylase activity DNA binding (Gu et al., 2002)
Pol λ	*human*	Van Loon and Hubscher, 2009; Markkanen et al., 2012c 40–130 (Dorn et al., unpublished results)	n.d.
PCNA	*human*	505–527 (Parker et al., 2001), F518/F519 (Chang and Lu, 2002)	n.d.
	S. pombe	438–445 (Chang and Lu, 2002)	n.d.
9-1-1	*human*	295–350 (Shi et al., 2006) V315, E316 (Shi et al., 2006; Luncsford et al., 2010)	Glycosylase activity (Chang and Lu, 2005), interaction increased after IR (Shi et al., 2006)
	S. pombe	245–293 (Chang and Lu, 2005) I261, E262	Glycosylase activity (Chang and Lu, 2005), interaction increased after H_2O_2 treatment
RPA	*human*	6–32 (Parker et al., 2001)	n.d.
ATR	*human*	n.d.	Checkpoint mediator? (Hahm et al., 2011)

n.d., not determined.

activity of *S. pombe* MUTYH was found with human Hus1 and the *S. pombe* 9-1-1. Human MUTYH was also observed to co-localize with Rad9 in cells treated with H_2O_2, suggesting that BER by MUTYH could be modulated by 9-1-1. Further work in *S. pombe* showed a decrease in repair of oxidative DNA damage *in vivo* when the interaction of MUTYH with 9-1-1 was disrupted, suggesting that this interplay significantly contributes to the response to oxidative stress (Luncsford et al., 2010). Also, MUTYH could be co-immunoprecipitated with ATR from human cells, possibly implicating MUTYH in ATR-mediated checkpoint execution (Hahm et al., 2011).

MUTYH from *S. pombe* was found to interact with Hst4, a histone deacetylase involved in silencing of genes and maintenance of genomic integrity, which seemed to regulate the levels of Hst4 after oxidative stress (Chang et al., 2011). Hst4 was further shown to interact also with the 9-1-1 complex. The association of MUTYH with telomeres was increased after oxidative stress and by deletion of Hst4, and Hst4 bound to telomeres decreased after oxidative stress, concomitant with a decrease in total Hst4 levels. Finally, MUTYH association with telomeres was increased in a Hst4 deletion background in the presence of chronic DNA damage caused by the lack of Hst4. Therefore, MUTYH seemed to regulate repair of telomeres by orchestrating the functions of 9-1-1 and Hst4. Finally, the WRN helicase/exonuclease was recently shown to promote MUTYH-initiated LP-BER of A:8-oxo-G mismatches by pol λ (Kanagaraj et al., 2012).

REGULATION OF MUTYH

REGULATION OF MUTYH LEVELS

So far, only a limited amount of studies has been performed concerning the regulation of MUTYH levels. Respiratory hypoxia caused a strong increase in mtDNA damage and also in expression of MUTYH mRNA in rat brain (Englander et al., 2002). This suggested that the increase denoted an adaptive mechanism for protection of neuronal DNA from oxidative injuries stemming from an imbalance in metabolism. Follow-up work by the same group identified specific MUTYH isoforms exclusive to brain tissue in rats, that were targeted to the mitochondria and some of them were inducible upon respiratory hypoxia (Englander et al., 2002). The divergence in the N-terminus between the different MUTYH isoforms was found to influence their excision rates and the processing of AP sites (Ma et al., 2004). In mononuclear blood cells MUTYH levels were neither altered by hypoxia nor by inhalation of 10% oxygen for 2 h and the subsequent reoxygenation period in healthy human subjects, even though DNA strand breaks and oxidatively damaged purines accumulated by this treatment (Risom et al., 2007). MUTYH, together with SMUG1, was regulated transcriptionally by p73, a member of the p53 protein family, through DNA damage induction by bile acid exposure, suggesting that this interplay regulates DNA damage repair (Zaika et al., 2011).

A comparison of embryonic stem cells to more differentiated cells did not reveal any impact on the mRNA levels of MUTYH, in contrast to OGG1, which decreased upon differentiation (Kuboyama et al., 2011). Alimentary supplementation with quercetin, a plant-derived flavonoid that has been attributed with anticarcinogen, was found to enhance the expression of MUTYH in the distal colon mucosa of rats (Dihal et al., 2008). And finally, overexpression of hepatitis B virus X (HBx) was shown to increase 8-oxo-G levels in HepG2 cells, and to decrease the transcript levels of MUTYHα mRNA, while not affecting mRNA of OGG1, suggesting that this may be linked to the development of hepatocellular carcinoma which is associated to HBx infection (Cheng et al., 2009).

REGULATION OF MUTYH BY POSTTRANSLATIONAL MODIFICATIONS

Very little is known about the regulation of MUTYH by posttranslational modifications (PTM) (**Table 3**). Findings from Gu et al. showed that MUTYH could be phosphorylated *in vitro* by different protein kinases (Gu and Lu, 2001). Comparison of the activity of native MUTYH from human cell extracts with in recombinant MUTYH purified from bacteria revealed a dramatic difference in the glycosylase activity, probably due to the phosphorylation state of the proteins. Indeed, the dephosphorylation of native MUTYH led to a tremendous loss of function on A:G or A:8-oxo-G mismatch containing templates. Differences in activity were also described for recombinant MUTYH expressed in bacteria or insect cells (Kundu et al., 2010). Mass spectrometric analysis confirmed S524 to be phosphorylated in the more active MUTYH, expressed in insect cells. Further functional studies using wt, phosphomimetic, or phosphodeficient mutants revealed an important role of S524 in substrate recognition and binding to DNA.

A defect in phosphorylation of MUTYH was also found to cause a mutator phenotype in different microsatellite stable colorectal cancer cell lines (Parker et al., 2002). All tested cell lines that showed elevated 8-oxo-G levels showed a decline in repair of A:8-oxo-G mismatches. While the sequencing of the *MUTYH* locus in these cells did not reveal any mutations, the mRNA and protein levels of MUTYH were decreased. In a subsequent study the same authors could demonstrate that a loss of MUTYH phosphorylation by PKC was responsible for the observed increase in 8-oxo-G causing the mutator phenotype (Parker et al., 2003). The 8-oxo-G repair capacity in MUTYH impaired cell extracts could be restored by complementation with PKC, PKA or casein kinase II. Furthermore, the same effect could be achieved by treatment with the PKC activator phorbol-12-myristate-13-acetate (PMA). In contrast to that, no effect in cell extracts from MUTYH proficient cells occurred, indicating that MUTYH was already

Table 3 | Posttranslational modifications of MUTYH.

Posttranslational modification	Site of modification	Kinase	Stimulatory effect
Phosphorylation (Gu and Lu, 2001)	n.d.	n.d.	Glycosylase activity
Phosphorylation (Parker et al., 2002, 2003)	n.d.	PKC PKA Casein Kinase II	Glycosylase activity
Phosphorylation (Kundu et al., 2010)	S524	n.d.	DNA-binding

n.d., not determined.

phosphorylated at a basal level in these cell lines. Consistent with these findings, MUTYH was a substrate for PKC *in vitro*. Finally, MUTYH purified directly from cell extracts treated with PMA showed an elevated capacity in the repair of A:8-oxo-G mismatches. So far it has not been elucidated whether phosphorylation only interferes with the catalytic activity of MUTYH, regulates its interaction with other proteins, or leads to a different subcellular localization. Since PKC can be stimulated by oxidative stress (Klein et al., 2000), it is possible that the phosphorylation-mediated regulation of MUTYH presents an adaptive response to DNA damage.

Taken together, it would be very interesting to investigate the regulation of MUTYH in more detail to get a better understanding how the different players of the 8-oxo-G repair machinery are controlled to protect cells from oxidative stress of endogenous or exogenous sources.

INVOLVEMENT OF MUTYH IN DISEASE

MAP (MUTYH ASSOCIATED POLYPOSIS)

Familial adenomatous polyposis (FAP) is an autosomal dominant disease characterized by the formation of hundreds to thousands of adenomatous polyps in the colons and rectums of the affected patients (reviewed in Fearnhead et al., 2001). It is caused by a germline mutation in the adenomatous polyposis coli (*APC)* gene, mutations that are also responsible for 80% of the sporadic colorectal tumors. In 2002, Al-Tassan and co-workers studied a British family with multiple colorectal adenoma and carcinoma, but failed to detect a mutation in the *APC* gene (Al-Tassan et al., 2002). Closer analysis of the patient material revealed an increased tendency of somatic CG→AT transversion mutations in the *APC* gene, which is consistent with 8-oxo-G mediated mutagenesis. This observation led the authors to dissect the three enzymes that work synergistically to counteract 8-oxo-G mediated mutagenesis, namely OGG1, MUTYH, and MTH. Sequencing of the respective genes revealed two non-conservative mutations in the *MUTYH* gene, Y165C (through an A→G transition) and G382D (through a G→A transition), while no pathogenic changes were observed in the *OGG1* and *MTH* genes. The two mutations were found to reduce the activity of *E. coli* mutY to remove A from G:A mismatches by 98% and 86%, respectively, suggesting that a defect in MUTYH activity was the reason for the accumulated mutations in the patients and thus responsible for the APC-like phenotype. Subsequent work revealed that these mutations not only compromise the bacterial mutY, but also caused a decrease in the activity of human MUTYH for excision of A opposite 8-oxo-G, which nicely correlated with the tumor phenotype (Al-Tassan et al., 2002; Chmiel et al., 2003; Pope and David, 2005). Further investigation led to the identification of seven other unrelated patients with colorectal adenomas or carcinomas that showed a bias of CG→AT transversion mutations to be carriers of biallelic germline mutations for MUTYH (Jones et al., 2002). This disorder is the only colorectal cancer form inherited in an autosomal recessive mode and is now commonly referred to as MAP, or infrequently also as FAP2 (http://www.omim.org). The prevalence of MAP is estimated to be around 1% of all colorectal cancer cases (Enholm et al., 2003; Croitoru et al., 2004; Fleischmann et al., 2004; Wang et al., 2004; Peterlongo et al., 2005; Webb et al., 2006; Kury et al., 2007; Cleary et al., 2009) and MUTYH mutations have been found in 7% (Filipe et al., 2009), and 10% (Pezzi et al., 2009) of FAP patients and 40% of AFAP patients, respectively (Filipe et al., 2009). The lifetime-cancer risk is assessed to 80% for colon cancer and 4% for duodenal cancer (Jasperson et al., 2010). Even though MAP is a rather recently discovered disease, many germline mutations in addition to the two found by Al-Tassan et al have been described so far. This is reflected in the abundance of literature investigating different single-nucleotide polymorphisms and their relevance to cancer development has been thoroughly reviewed in Cheadle and Sampson (2007) and Poulsen and Bisgaard (2008). Interestingly, other than MUTYH, no association of further genes involved in BER or the repair of oxidative DNA damage with a multiple colorectal adenoma phenotype has been found so far (Dallosso et al., 2008). Interestingly though, work by the Sweasy group has found that the *POLB* gene is mutated in many colorectal cancers, suggesting that at least some of these mutations may lead to compromised BER function in the affected tissues (Donigan et al., 2012; Nemec et al., 2012). MAP patients have been reported to be more prone also to extraintestinal tumors such as ovarian, bladder, skin, and breast cancer. For an overview of all extracolonic manifestations of MAP-patients, please refer to this recent review (Nielsen et al., 2011). For further clinical features, there are excellent recent reviews available (Jasperson et al., 2010; Nielsen et al., 2011). Several mutations in MUTYH associated with MAP were found to significantly enhance the spontaneous mutator phenotype of patient's lymphoblasts under conditions of oxidative stress and to accumulate 8-oxo-G in the DNA, underlining the role of MUTYH in the pathogenesis of this disease (Ruggieri et al., 2012). However, for many of the mutants it is unclear how the mutation affects its activity, and more work is needed to clarify their exact contribution to the disease.

EQUINE CEREBELLAR ABIOTROPHY

Interestingly, MUTYH has been suggested to be involved in the pathogenesis of equine cerebellar abiotrophy, a neurological disease found in Arabian horses, as indicated by a SNP in the GATA2 binding region of the MUTYH promoter (Brault et al., 2011). Whether there is a real causative role and what mechanisms are behind it, remains to be elucidated by further studies.

CONCLUSIONS AND PERSPECTIVES

The MUTYH DNA glycosylase is a remarkable enzyme since it has the specificity to remove an undamaged DNA base from a mismatch such as an A:8-oxo-G. It is found throughout evolution from bacteria to human, suggesting an essential role in preventing mutations arising from oxidative damage to the DNA. During the last three decades, our knowledge about how MUTYH functions has grown substantially. We now understand quite in detail how MUTYH acts catalytically, and the structures of prokaryotic and eukaryotic enzymes have been identified. However, the functional details of the at least 10 isoforms of MUTYH, are far from being unequivocally clarified. MUTYH acts together with pol λ in the so-called MUTYH/pol λ pathway that can replace a promutagenic A paired to an 8-oxo-G with a correct C. The interaction with the moving platforms PCNA and the 9-1-1

complex is apparently very important for the proper spatial and temporal engagement of MUTYH on the DNA, and there especially in the context of chromatin. So far, very little is known about the regulation of MUTYH, which is at least in part likely achieved by PTM. Phosphorylation as an important PTM contributes to regulate the activity of MUTYH. It is likely that other PTM's, such as ubiquitination, will be identified that govern the temporal (i.e., during the cell cycle) as well as the spatial (i.e., the subcellular localization) distribution of MUTYH. Also, the fact that mutations in MUTYH are identified in diseases of human and animals shifts this enzyme more and more into the focus of translational medicine. In the future, it will be of interest to understand more about the subcellular localization and specific functions of the different isoforms of MUTYH. Also, the exact regulation of the activity, stability, and localization of this enzyme is likely to yield many novel insights. Finally, we are anticipating further clarification of the functional roles of the different mutations in MUTYH associated with MAP.

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3.4.1. Recent developments according the MutYH glycosylase – an update since 02/2013

The polymorphic AluYb8 insertion in the 15th intron of the MutYH gene (AluYb8MutYH) was already known to increase the amount of 8-OH-dG lesions in the genomic DNA (80). In a follow up study Guo *et al.* could show that this variant leads to a significant reduction of the mitochondrial MutYH variant. AluYb8MutYH further affects the maintenance of mitochondrial DNA (mtDNA) and the functional mitochondrial mass in homozygous individuals (81). This result confirms an important role for MutYH in the repair of mtDNA.

MutYH interacts with the Rad9-Rad1-Hus1 (9-1-1) complex as described before (82). A recent study investigated the role of MutYH in DNA damage repair in the context of 9-1-1 depletion in yeast. Treatment of cells with cisplatin led to an overall accumulation of MutYH while phleomycine treatment caused a more specific increase of MutYH on the chromatin. Jannson *et al.* observed a general re-localization of MutYH to the nucleus after exposure of cells to chemotherapeutics (83).

A MutYH variant found in MAP patients, MutYH Q338H, was characterized by Turco *et al.* They found the glycosylase activity to be completely retained, but a reduced ability to bind the 9-1-1 complex. Cells expressing this mutant showed a hypersensitivity to oxidants and an accumulation in S-phase (84). Taken together these results show that a proper interaction between the S-phase checkpoint 9-1-1 and MutYH is needed to enable DNA damage repair.

Analysis of patients suffering from colorectal cancer elucidated that the 324His/His MutYH genotype is associated with a higher risk of cancer development. The authors could further correlate a decreased repair capacity of oxidative DNA damages with this MutYH mutation phenotype (85).

Consistent with this result another study identified a KRAS mutation in MAP patients. This mutation was not found in patients suffering from classical/attenuated familial polyposis adenomas. The c.34G>T transversion mutation in the KRAS gene is typically associated with oxidative DNA damage (86).

Ruggieri *et al.* analyzed lymphoblast cell lines derived from seven MAP patients, that carry different MutYH mutations. All of the tested cells were defective in removing A mispaired to 8-oxo-G and consequently showed elevated mutation rates.

The mutation frequency could even be enhanced by treatment with KBrO3 (72).

Taken together these recent results confirm again the important role of MutYH in the maintenance of genomic integrity and the consequences of any mis-regulation.

3.5. DNA polymerase λ

DNA Pol λ is, together with DNA Pol β, μ and TdT, a member of the Pol X family, which is known to be mainly involved in the repair of small gaps in the DNA (87). The protein is encoded on chromosome 10 and gives rise to a 575 kDa protein (88) that was shown to be expressed at the highest levels in testis, ovary and fetal liver (89,90).

The protein is structured in a BRCT domain (N-terminal-BRAC1 (breast-cancer-susceptibility protein-1) C-terminal-domain), a serine-prolin-rich region and in the C-terminal catalytic core (Figure 6) (91). The catalytic core of DNA Pol λ shares 33% of identity with DNA Pol β and consists of the 8 kDa domain and the polymerase domain, including palm, fingers and thumb. The BRCT domain is suggested to mediate protein-protein interactions while the serine-prolin-rich domain is most likely a target for PTM (89).

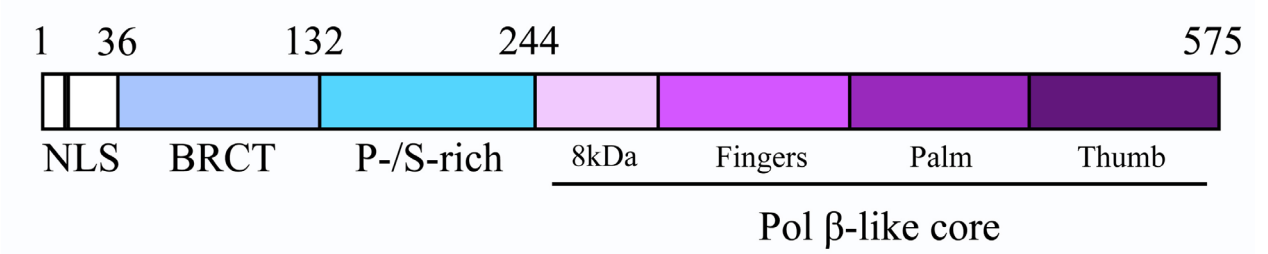

Figure 6: Linear diagram of the full length DNA polymerase λ.
Reproduced from (92) . For details see text.

DNA Pol λ was shown to be involved in BER, translesion synthesis (TLS) and non-homologous-end-joining (NHEJ) (1,89,93-97). The first result connecting DNA Pol λ with BER showed DNA Pol $\lambda^{-/-}$ mouse embryonic fibroblasts (MEF) to be hypersensitive against oxidative stress (98). A follow up study revealed that DNA Pol λ can serve in a backup BER mechanism in DNA Pol $\lambda^{-/-}$ cells (95). In 2007 Maga *et al.* found that DNA Pol λ, together with its co-factors PCNA and RP-A, incorporates the correct C about 1200 fold more efficient opposite a 8-oxo-G lesion than the wrong A (39), highlighting the crucial role of DNA Pol λ in the repair of oxidative DNA damage. PCNA and RP-A act as a molecular switch to allow the faithful repair of 8-oxo-G by DNA Pol λ and block at the same time the recruitment of DNA Pol β to the lesion (99).

DNA Pol λ was shown to be able to perform TLS of an AP site following the synthesis by DNA Pol ε up to the this site (97). Additionally DNA Pol λ can help to bypass a

8-oxo-G:C mispair (100). Since DNA Pol δ pauses at this site a switch to DNA Pol λ is required to overcome the lesion.

The role of DNA Pol λ was also confirmed *in vivo* showing that DNA Pol λ together with MutYH localizes at the sites of DNA damage allowing the MutYH initiated LP-BER to take place (40). The recruitment of DNA Pol λ was shown to be regulated by interplay between PTM (67) and will be discussed in detail in the following. Consistent with its predicted role as repair factor in the S-phase of the cell cycle the DNA Pol λ level were found to be highest in late S and G2 phase.

Maga e*t al.* could recently show that the activity DNA Pol λ is also required for the cell cycle progression (101). Knock down of DNA Pol λ led to replication fork stress and S phase checkpoint activation, what can have detrimental consequences for the cell.

3.6. Ubiquitination

3.6.1. Ubiquitin as posttranslational modification

Since its discovery in the late 1970s (102) the role of ubiquitination has been studied intensively and was mainly associated with the targeting of misfolded proteins to proteasomal degradation. In the mean time a lot of studies were published reporting that ubiquitin modulates many other cellular processes like DNA repair (103), receptor endocytosis (104,105), apoptosis (106) and autophagy (107).

Ubiquitin is a 76 amino acid peptide that is attached reversibly to its substrate by an isopeptide or amide bond. Ubiquitin is mainly attached to K residues of the target protein but recent results showed that also the binding to free α-NH_2 groups (108) or cysteine residues (109) is possible. Ubiquitin can be attached either as mono-, di- or polymer. The linkage between the single moieties can be mediated by all seven K residues present in ubiquitin: K6, K11, K27, K29, K33, K48 and K63, whereas K48 and K63 are the best characterized (110).

Ubiquitin is synthesized as inactive precursor protein (111). Before an ubiquitin moiety can be attached to a substrate it needs to be activated in an ATP dependent reaction that is carried out in a three enzyme based reaction. The ubiquitin-activating enzyme (E1) adenylates ubiquitin at the C-terminus, leading to the formation of a high reactive thioester bond. The thioester is further transferred to the ubiquitin-conjugating (E2) enzyme and finally the ubiquitin ligase (E3) specifically attaches the ubiquitin to the

respective substrate (Figure 7) via an isopeptide linkage between the C-terminus and the ε-amino-group of the K residue. The deubiquitination of proteins is mediated by another class of enzymes, the so called deubiquitinases (DUB) that cleave isopeptide bonds.

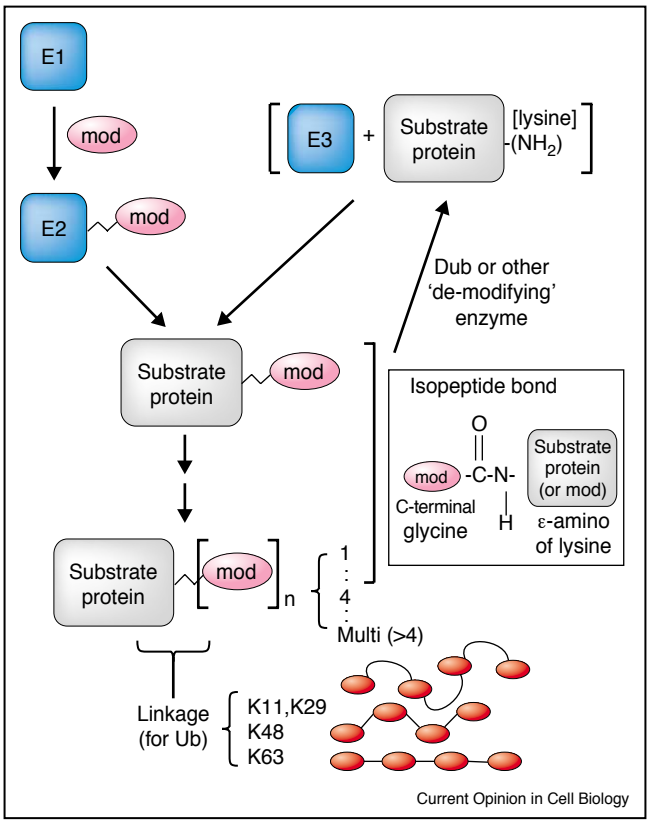

Figure 7: Overview of the ubiquitination cycle. Reproduced from (112). For details see text.

The substrate specificity in this process is given mainly by the E3's, what is also reflected by the variety of this enzyme class. While just two E1's and about 30 E2's, more than 600 E3 enzymes were identified so far in humans.

There are two main classes of E3 ligases, the homologous to the E6AP carboxyl terminus (HECT) and the really interesting new gene (RING) domain containing ligases. Two zinc ions in the RING domain bind the substrate and the E2 enzyme and bring them together to facilitate the ubiquitin transfer. In contrast to the HECT domain containing E3 ligases these ones act just as an adaptor of the reaction. The HECT domain contains an N-terminal active cysteine (C) residue to that the ubiquitin is transferred. The substrate binds to the C-terminus of the domain and is brought together with the ubiquitin by movement of the HECT domain (113). To get the two parts of the domain in close proximity movements of up to 50 Å are required (114).

Depending on the length and the linkage of the ubiquitin chains the modification can have different consequences for the target. Mono-ubiquitination was shown to be mainly involved in endocytosis and transcriptional regulation (113). Ubiquitin linked via K63 is involved in cellular processes such as DNA repair and signal transduction (115),

while ubiquitin attached by K48 leads to the proteasomal degradation, if the chains have a length of at least four ubiquitin moieties (116).

The proteasome degrades ubiquitinated proteins and releases ubiquitin moieties and peptide fragments. It is a huge protein complex consisting of 50 subunits and has a total size of 2.4 mDa. The 26S proteasome is composed of the 20S and the 19S subunits. The 19S subunit is mainly responsible for the recognition of ubiquitin chains. After its binding the substrate is transported in the core particle. The 20S subunit is built up of four stacked rings that form a tube like structure containing the proteolytic chamber in the middle. In the active core the substrate can either be cleaved after hydrophobic, acidic or basic residues thereby guaranteeing the degradation of almost every peptide sequence (117).

Ubiquitination not only causes proteasomal degradation but was also shown to regulate the subcellular localization of a protein. The localization of the MEK1 kinase is modulated by crosstalk of ubiquitination and SUMOylation (small ubiquitin like modifier). While the latter leads to re-localization of MEK1 from the nucleus to the cytoplasm, ubiquitination maintain the enzyme inactive and kept in the nucleus (118). The opposite effect was observed for the von Hippel-Landau tumor suppressor protein (119). The SUMOylated protein stays predominantly in the nucleus whereas ubiquitination causes cytoplasmic localization and protein degradation.

Taken together ubiquitination is a powerful tool not only to regulate the levels of a protein, but also its function and localization.

3.6.2. Ubiquitination in base excision repair

Several reports already link ubiquitination with DNA damage repair. Besides its role in double strand break repair (DSB) (reviewed in (120)) and TLS (reviewed in (121)) recent reports established ubiquitination to be an important regulatory mechanism in BER. Mule was identified to be the E3 ubiquitin ligase responsible for the ubiquitination of different members of BER.

Upon exposure to oxidative stress DNA Pol β gets monoubiquitinated by Mule (122), what marks the protein for polyubiquitination and subsequent proteasomal degradation (123). Dianov *et al.* could show that, as long as DNA Pol β, XRCC1 and DNA ligase III are parts of an active repair complex on chromatin, they are protected from

ubiquitination (123). Thereby a mechanism is guaranteed how the cell can modulate the levels of repair proteins as a function of the amount of DNA damage.

For DNA Pol λ the regulation was shown to depend on the interplay between phosphorylation and ubiquitination. DNA Pol λ is a target of the CDK2/cyclin A kinase (124), that protects the protein from becoming ubiquitinated by Mule (67) and subsequently degraded. Thus DNA Pol λ is stabilized in late S- and G2 phase of the cell cycle, at the time when the repair capacity of DNA Pol λ is needed most.

Consistently, Mule itself is regulated in a DNA damage dependent manner. One regulator of Mule is the alternative reading frame (ARF) protein that accumulates upon oxidative stress and leads to the degradation of Mule (125). A second, ARF independent regulation mechanism for Mule was recently discovered involving an isoform of the ubiquitin specific processing protease 7 (USP7), USP7S (126). Upon exposure of cells to oxidative stress USP7S is down regulated and favors thereby the self-ubiquitination of Mule leading to degradation.

These regulation mechanisms ensure, that as consequence of DNA damage the E3 ligase Mule is either inactivated by ARF or degraded and BER enzymes thus are stabilized to act at the sites of damage. Consequently DNA repair leads to a reduction in ARF and an elevated Mule activity (Summarized in Figure 8). A tight regulation is urgently needed since BER activity is continuously required and the cell must react quickly to small changes in the amount of DNA damage to prevent deleterious mutations.

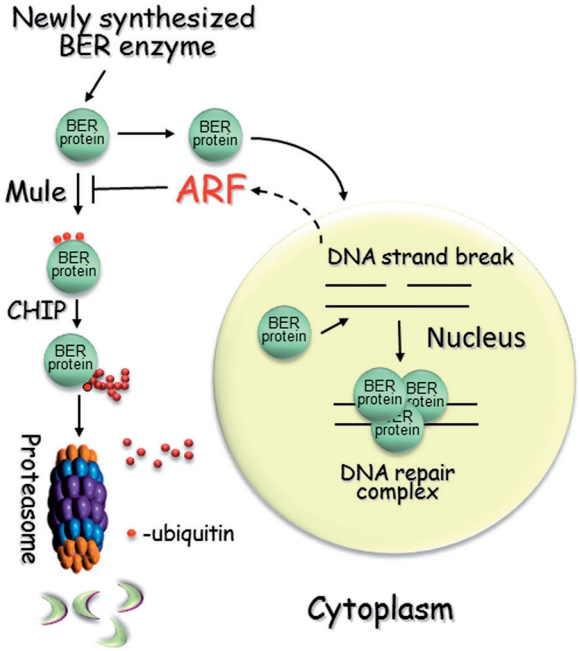

Figure 8: Regulation of steady state levels of BER enzymes by Mule.
Reproduced from (127). For details see text.

3.7. The E3 ligase Mule

Mule was shown to be involved in many cellular processes as proliferation (128), DNA replication (129) and DNA repair (67,122).

It is a member of the HECT domain containing E3 ligases (128). The catalytically active domain of the 482 kDa protein is located at the C-terminal 350 amino-acids. A crystal structure (Figure 9) (130) of Mule revealed that the HECT domain itself is distributed in two subdomains that are connected by a flexible linker. One part contains the active C residue while the other one is responsible for binding the E2 enzyme. Further, the HECT domain contains a single α-helix, that was shown to be indispensable for the stability of the enzyme and its knock down enhances the self ubiquitination of Mule. This fact can be explained by the higher flexibility of the protein facilitating the ubiquitination reaction.

The N-terminal domains of Mule are involved in interactions with other proteins. The ubiquitin associated domain (UBA) binds ubiquitin chains (131) while the WWE (132) and the BH3 domain are believed to mediate the binding of interaction partner. The function of the two armadillo repeat like (ARLD) domains remains so far elusive (133).

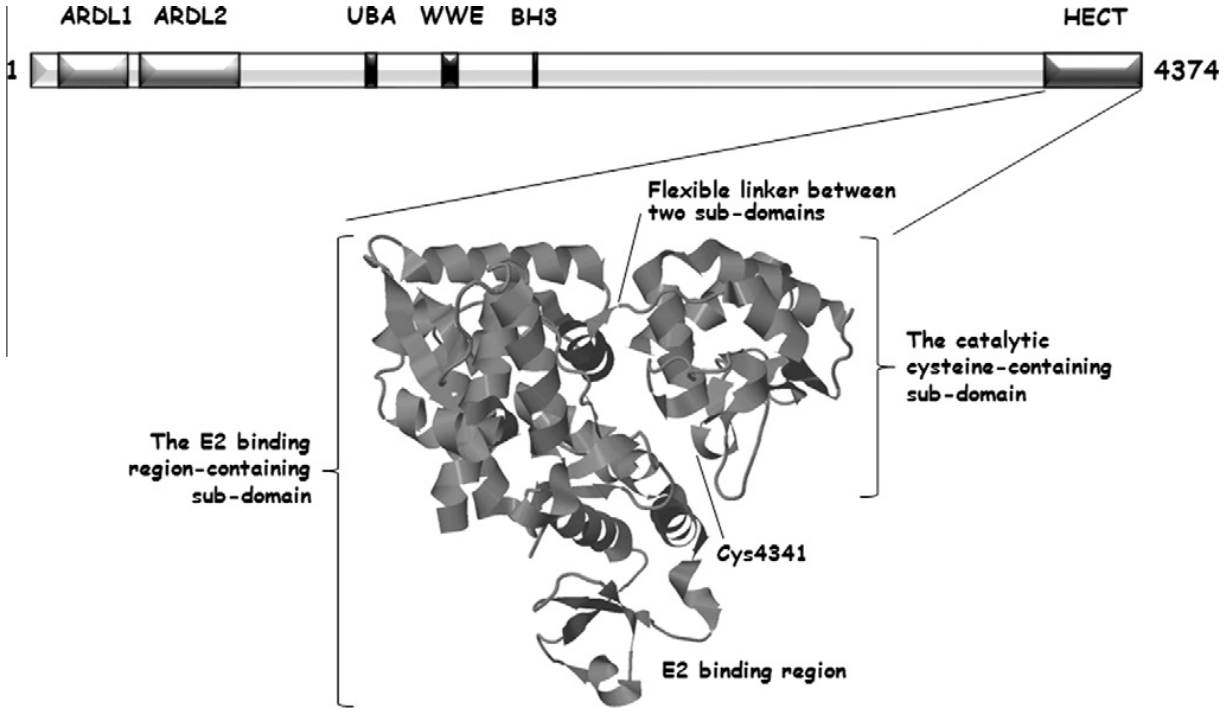

Figure 9: Domains of the Mule E3 ligase and structure of its HECT domain.
Reproduced from (134). For details see text.

4. Original research article: "Regulation of Human MutYH DNA Glycosylase by the E3 Ubiquitin Ligase Mule"

Julia Dorn, Elena Ferrari, Ralph Imhof, Nathalie Ziegler and Ulrich Hübscher

In the following manuscript, in which I am the first author, the regulation of the DNA glycosylase MutYH by ubiquitination was identified. Further a dependency of the mutation frequency on the amount of MutYH in cells was found.

The manuscript is currently under review.

Regulation of Human MutYH DNA Glycosylase by the E3 Ubiquitin Ligase Mule

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ABSTRACT

Oxidation of DNA is a frequent and constantly occurring event. One of the best-characterized oxidative DNA lesions is 7,8-dihydro-8-oxoguanine (8-oxo-G). It instructs most DNA polymerases to preferentially insert an adenine (A) opposite 8-oxo-G instead of the appropriate cytosine (C) thus showing miscoding potential. The MutY DNA glycosylase homologue (MutYH) recognizes A:8-oxo-G mispairs and removes the mispaired A giving way to the canonical base excision repair that ultimately restores undamaged guanine (G). Here we show that human MutYH is ubiquitinated *in vitro* and *in vivo* by the E3 ligase Mule. The MutYH levels depend on the amount of expressed Mule. Furthermore MutYH and Mule physically interact. Ubiquitination of MutYH occurs between amino-acids 475-535. Mutation of the five lysines in this region, significantly stabilizes MutYH, suggesting that these are the amino-acids used for ubiquitination of MutYH. We found that an ubiquitination-deficient MutYH mutant showed enhanced chromatin binding. The mutation frequency of the ovarian cancer cell line A2780, analyzed at the HPRT locus was dependent on the Mule levels and could be increased upon oxidative stress.

In summary our data show that ubiquitination is an important regulatory mechanism for the essential MutYH DNA glycosylase in human cells.

INTRODUCTION

Every organism is exposed to high levels of oxidative stress every day, due to a variety of endogenous and exogenous sources. One of the most often observed consequences is the formation of the highly mutagenic lesion 7,8-dihydro-8-oxoguanine (8-oxo-G), that arises approximately 10^3 times per cell and day in normal tissue and up to 10^5 times in cancer tissues (1). The 8-oxo-G lesion is potentially highly mutagenic, since replicative Pols tend to incorporate the incorrect A rather than the correct C opposite the lesion, leading to the formation of an A:8-oxo-G mispair. If these mispairs are not repaired before the next replication cycle, GC->TA transversion mutations occur. Such mutations

were found to be frequently present in different types of cancer tissues (2), demonstrating the paramount importance for the organism of a mechanism ensuring the correct repair of 8-oxo-G lesions. As we have previously shown (3, 4), such a mechanism indeed exists. It is a specialized base excision repair (BER) pathway coordinated by the glycosylase MutYH and DNA polymerase (Pol) λ. MutYH recognizes a mispaired A and excises it, leading to the formation of a one nucleotide gap, still bearing the 8-oxo G lesion on the template strand, that is subsequently filled by Pol l. In contrast to other Pols, Pol λ and to some extent Pol η, both in the presence of proliferating cell nuclear antigen (PCNA) and replication protein A (RP-A), are very efficient in the correct bypass of an 8-oxo-G lesion thereby preventing the generation of mutations (3).

In agreement with the important role of MutYH in the repair of oxidative DNA damage, mouse embryonic fibroblasts as well as embryonic stem cells derived from MutYH $^{-/-}$ mice show an increase in mutation frequency (4,5). Furthermore, knockout mice have a higher incidence of tumor formation compared to the wild type mice. This effect can even be increased by exposing the mice to an oxidant showing that the absence of MutYH drastically impairs the repair of oxidative damage (6). Intriguingly, MutYH transcript levels are inversely correlated with the survival outcome of patients suffering from gastric cancer (7).

The BER machinery needs to be tightly controlled to ensure an efficient and correct repair of damaged DNA. Already small differences in expression of individual BER proteins can disturb the entire pathway and thus lead to a reduced repair capacity (8). Recent data provide evidence that regulation of BER is mainly achieved through various posttranslational modifications (PTMs), such as phosphorylation and ubiquitination (9-12). The steady state levels of the BER members, Pol β and Pol λ, are tightly controlled by ubiquitination leading to proteasomal degradation, in order to guarantee the correct repair of damaged DNA (reviewed in (13)) . The E3 ubiquitin ligase responsible for the ubiquitination of Pol β and Pol λ, was identified to be Mule, a HECT domain containing E3 ligase involved in many cellular processes like cell proliferation, DNA replication and repair (14). On the other hand, very little is known about the PTMs of MutYH and their potential roles in the regulation of the protein. Only phosphorylation was shown to stimulate the glycosylase activity of MutYH (15).

In the present paper we provide the first evidence that MutYH is also a target for ubiquitination by the E3 ligase Mule. Ubiquitinated MutYH is marked for proteasomal degradation thereby not only modulating the protein level itself but also the intracellular distribution of the protein. We furthermore show that a tight regulation of MutYH is of great importance, since altered protein levels lead to an increase in mutation frequency at the HPRT locus of the ovarian cancer cells A2780.

MATERIAL AND METHODS

Chemicals

Oligonucleotides for site directed mutagenesis were purchased from Microsynth and $KBrO_3$ from Sigma. The Mule siRNA (Hs_HUWE2) was purchased from Qiagen.

Cells and Extracts

HeLa cells were purchased from American type cell culture. HEK293T and A2780 (described in ref. (16)) cells were gifts from R. Santoro (University of Zürich, Switzerland), and J. Jiricny (University of Zürich, Switzerland), respectively. Cells were grown under standard conditions. Whole cell extracts were prepared by scraping cells into lysis buffer (10% glycerol, 1% Triton X-100, 1,5 mM $MgCl_2$, 50 mM Hepes (pH 7,5), 150 mM NaCl, 1 mM EGTA, 100 mM NaF, 10 mM $Na_2P_2O_7$, 1 mM Na_3VO_4, 1 mM PMSF, 1 µg/ml bepstatin, 1 µg/ml leupeptin, 1 µg/ml pepstatin) and incubated for 5 min on ice. The cell lysates were sonicated at 4°C for 2.5 min
and centrifuged for 10 min at 15.000 g. The supernatants were collected and stored at -80°C.
Cell fractionations into cytoplasmic, nuclear and chromatin fractions were performed as described in ref. (10).

Western Blot Analysis

Western Blot analysis was performed according to standard protocols and visualized by the Odyssey image analysis system (Li-cor, Bioscience).

Antibodies and Proteins

The antibodies (AB) against MutYH and GST were purchased from St. Cruz. The Mule AB was from Bethyl Laboratories, the tubulin AB from Sigma-Aldrich and the Histone H3 AB from Abcam. The Flag AB was from Sigma, the HA AB from Covance, the His AB from Qiagen and the Actin AB from Sigma. Recombinant MutYH was expressed and purified as described in ref. (17) and the HECT domain of Mule according to ref. (12).

RNA Interference

Cells were transfected using Lipofectamin RNAiMax (Invitrogen) according to the manufacturer`s instructions and harvested 72h after transfection.

Site Directed Mutagenesis

Site directed mutagenesis was performed using *PfuTurbo* Pol from Stratagene according to the manufacturer`s instructions. The primers (Microsynth) were designed using PrimerX.
MutYH KK477RR:5-GTTTCCACCGCCATGAGAAGGGTTTTCCGTGTGTATC-3
and 5-GATACACACGGAAAACCCTTCTCATGGCGGTGGAAAC-3,

K495R: 5-CCTGTATGGGTTCCAGAAGGTCCCAGGTGTC-3

and 5-GACACCTGGGACCTTCTGGAACCCATACAGG-3,

KK506RR: 5-CCGTGCAGTCGGAGAAGGCCCCGCATGGGCC-3

and 5-GGCCCATGCGGGGCCTTCTCCGACTGCACGG-3 ;

HECT C326A: 5-CTGCCTTCAGCTCACACAGCTTTTAATCAGCTGGATC-3

and 5-GATCCAGCTGATTAAAAGCTGTGTGAGCTGAAGGCAG3. The primers were used on the pcDNA3 HA-MutYH and the pcDNA3 Flag-MuleΔN447 plasmids and the mutagenesis was confirmed by sequencing.

qPCR

Total RNA was isolated from cells using the Nucleo Spin RNAII Kit (Macherey & Nagel), RT-PCR was performed using the QIAGEN OneStep RT-PCR Kit. qPCR was performed using SYBR Green (Qiagen), 150 ng of total RNA and the oligonucleotides (Microsynth) as follows: HA-MutYH: 5-GCCAGCAAGTCCTGGATAAT-3 and 5-ATGCGTAGTCAGGCACGTC-3 ,MutYH: 5-CCAGAGAGTGGAGCAGGAAC-3 and 5-TTTCTGGGGAAGTGGACCAC-3 ,L28: 5-GCAATTCCTTCCGCTACAAC-3 and 5-TGTTCTTGCGGATCATGTGT-3. L28 was used as internal standard.

$KBrO_3$ Treatment and Cell Based Mutagenesis Assay

The cell based mutagenesis assays were performed using the A2780 cell line as described in ref. (16) Briefly the cells were transfected with plasmid or siRNA as indicated in the Figure, 24h later the cells were seeded for treatment with $KBrO_3$ (concentrations as indicated, 30 min). After culturing the treated cells for 7 days, they were reseeded and cells harboring a mutation in the HPRT1 gene were selected by adding 6-TG (5 µg/ml, Sigma). The mutation rate was calculated after 7 days under selection, the number of colonies was normalized to the seeding efficiency as described in (16).

GST Pull-Down Assay

The GST pull-down assay was performed using recombinant and purified GST-tagged MutYH (2 µg) coupled to GST-sepharose beads and incubated with 800 ng of recombinant HECT-domain of Mule. The assay was performed as described in detail in ref. (17).

Pull-Down Assay

Cell extract containing overexpressed flag tagged HECT domain of Mule was incubated with flag AB (1 µg) and 25 ml of protein G sepharose beads (GE Healthcare) for 2 h. After washing three times with a buffer containing PBS with 0.2% Triton, X-100, 1 mM DTT, 1 mg/ml leupeptin, 1 mg/ml pepstatin, 1 mg/ml bestatin, and 1 mM PMSF 800 ng of recombinant purified protein was added to the beads. After three times washing the beads were finally resuspended in Laemmli buffer and the samples were analyzed by Western blotting.

Detection of Protein Ubiquitination

The protocol was used to eliminate all non-covalent protein binding and was performed as described in detail in ref. (18).

Co-Immunoprecipitation

Cell extracts were prepared as described, 1:2 diluted in HNTG buffer (20 mM Hepes (pH 7.5) 150 mM NaCl, 10% glycerol 0,1% Triton X-100, 10 mM NaF, 1 mM Na_3VO_4) and incubated with 2 µg AB as indicated and 25 ml of protein G sepharose beads. After rotating for 2 h at 4°C the beads were washed three times with HNTG buffer, finally resuspended in Laemmli Buffer and analyzed by western blotting.

***In vitro* Ubiquitination Assay**

In vitro ubiquitination was performed as described in ref. (12). Briefly the assay was performed in a 15 µl reaction mixture containing E1 activating enzyme (0.7 pmol), E2 conjugating enzymes (9.5 pmol) and ubiquitin (0.6 nmol, Boston Chemicals). The reaction was carried out in buffer containing 25mM Tris–HCl (pH 8.0), 4 mM ATP, 5 mM $MgCl_2$, 200 mM $CaCl_2$, 1 mM DTT, 10 mM MG132 for 1 h at 30°C. Finally, Laemmli buffer was added and the samples were analyzed by western blotting.

DNA Isolation and 8-oxo-G Determination

Approximately $2x10^7$ cells were harvested and immediately stored at -80°C. The DNA was isolated with the DNA Isolation Kit for Cells and Tissues (Roche) according to the manufacturers instructions. The 8-oxo-G determination was carried out following the protocol depicted in ref. (19).

Microscopy

HeLa cells were transfected with plasmids as indicated using Lipofectamin2000 (Invitrogen) according to the manufacturer`s instructions. The cells were seeded on glass coverslips and fixed using methanol for 20 min at -20°C. After blocking with PBS/10%FCS the cells were incubated with the indicated primary AB (1:300 in PBS/10%FCS) for 2 h. The coverslips were washed three times with PBS/10%FCS (30 min), incubated with the secondary Cy3 (1:300 in PBS/10%FCS) for 1h, washed three times and fixed on object slides using Vectashield (Vector labs). The pictures were taken using the Leica CTR 6000.

RESULTS

MutYH is Ubiquitinated *in vitro* and *in vivo* by the E3 Ubiquitin Ligase Mule.

Former studies identified the E3 ubiquitin ligase Mule as a regulator of the steady state levels of different proteins involved in BER (10,12,20). Here, we were aiming at the identification of the regulatory mechanisms controlling the MutYH protein levels in the cell. Using an *in vitro* ubiquitination approach we tested whether MutYH was a substrate for the E3 ubiquitin ligase Mule. Mule is a 482 kDa protein whose catalytical center lies in the homologous to the E6-AP carboxyl terminus (HECT) domain, mapped to the C-terminal 370 amino acids of Mule (21). Using the recombinant, purified HECT domain in an *in vitro* ubiquitination assay (Figure 1 A), we found that MutYH got ubiquitinated by the HECT domain in presence of the E2 conjugating enzymes H5b, H5c and H7. In order to get more distinct bands a mutant ubiquitin, not able to form poly-ubiquitin chains, was used in all *in vitro* assays.

We next addressed the question whether Mule also plays a role in the regulation of MutYH *in vivo.* We transfected a N-terminal deletion construct of Mule containing the full HECT domain (Mule ΔN2474) together with MutYH for overexpression into HEK293T cells. As shown in Figure 1 B the co-transfection led to the appearance of higher migrating MutYH bands (lane 2 and 3) in contrast to the control (lane 1) in which just one form of MutYH was present. The fact that addition of the proteasomal inhibitor MG132 (lane 3) to the transfected cells prior to harvest caused an increase in the intensity in the higher migrating bands of MutYH, further suggested that these bands represented an ubiquitinated form of the protein. To ensure that the catalytic activity of Mule was required for the modification of MutYH, the experiment was repeated using a catalytic dead mutant of Mule (Mule C4341A). In contrast to the first experiment using the Mule WT, the co-transfection of the Mule C4341A mutant together with MutYH did not result in higher migrating forms of MutYH (Figure 1 C).

We performed a transient KD of Mule in HEK293T cells (Figure 1 D) that overexpressed MutYH and ubiquitin. After inhibition of the proteasomal degradation by adding MG132 we purified MutYH by binding to beads and analyzed the amount of ubiquitinated MutYH (Figure 1 E). The KD of Mule resulted in a 50% reduction of modified MutYH, indicating that Mule is the enzyme responsible for ubiquitination of MutYH (Figure 1 F). The observed dramatic decrease in the formation of ubiquitinated MutYH upon knock down (KD) of Mule, further supports its role as the E3 ligase for MutYH ubiquitination.

MutYH Protein Levels Depend on the Amount of the E3 Ubiquitin Ligase Mule.

To show the relevance of regulation by Mule *in vivo*, we performed a transient KD of Mule using siRNA expecting to see an effect on the endogenous protein levels of MutYH. As shown in Figure 2 A and B the KD of Mule led to a significant increase in MutYH protein level, while neither the treatment with Lipofectamine nor with scrambled (scr) siRNA showed this effect. Unchanged mRNA levels of MutYH upon Mule KD were indicative that the regulation of MutYH was at the protein level (Fig. 2 C).

Consistent with these results we observed the opposite upon Mule overexpression. The transfection of HEK293T cells with Mule caused a dramatic decrease in the endogenous level of MutYH compared to the empty vector control (Figure 2 D and E). As expected the MutYH mRNA level did not change upon overexpression of Mule (Figure 2 F). These results led us to conclude that indeed Mule is the E3 ubiquitin ligase responsible for the regulation of MutYH on protein level *in vivo*.

MutYH and Mule Physically Interact.

The experiments shown above, while strongly suggesting that Mule binds to and ubiquitinated MutYH, do not exclude the possibility that Mule may function as scaffold protein, bringing MutYH together with other proteins, ultimately responsible for its ubiquitination. We then addressed the question whether MutYH and Mule directly interact. In the first approach we could confirm the physical interaction between the two proteins by performing pull-down assays using tagged, recombinant and purified proteins. The pull down of GST-tagged MutYH also enriched the HECT domain demonstrating its direct interaction with MutYH (Figure 3 A). Moreover, the pull-down of the HECT domain using flag-beads confirmed the interaction between the two proteins (Figure 3 B).

Finally we showed that the interaction could be also recapitulated in cells. We performed co-immunoprecipitation experiments using whole cell extract containing overexpressed flag-tagged Mule and HA-tagged MutYH. By binding of Mule to flag-beads we could show, that MutYH co-immunoprecipitated (Figure 3 C). The interaction could be also confirmed *vice versa* by coupling MutYH to HA-beads and analyzing the samples for co-immunoprecipitated Mule (Fig. 3 D), strengthening the point that Mule directly ubiquitinates MutYH.

MutYH is Ubiquitinated Between Amino-acids 475-535 and Mutation of the Five Lysine Residues in this Part of the Protein Stabilize MutYH on the Protein Level.

Ubiquitin chains are covalently attached to lysine residues on the target protein. The sequence of MutYH reveals a total of 17 lysine residues distributed all over the protein, with an accumulation on the C- and N-terminal sites. In order to identify the lysine residues that are the targets for ubiquitination by Mule we generated deletion constructs of MutYH (Figure 4 A): one construct spanning the N-terminal part (amino-acids 1-350) containing the glycosylase domain of MutYH, the other construct consisting of the C-terminal MutT-like domain (amino-acids 350-535). Using these constructs in an *in vitro* ubiquitination approach we could identify the MutT like domain of MutYH (lane 6) to be ubiquitinated by Mule, while the N-terminal part or MutYH was not modified (lane 4, Fig. 4 A). To narrow down the region we generated further deletion constructs of the MutT-like domain. Once the amino-acids 475-535 were deleted the MutYH construct was not ubiquitinated any more (lane 12 and 14) leading finally to the conclusion that the C-terminal 60 amino-acids of MutYH contain the ubiquitination sites. The sequence of MutYH (Figure 4 B) contained five lysine residues within this region as potential targets for ubiquitination (Figure 4 B). To address the question which

function these lysine residues have for the regulation of MutYH we generated ubiquitination deficient, lysine to arginine point mutants: KK477RR, K495R, KK506RR and one construct containing all five lysine residues mutated to arginine (5R) (Figure 4 B). Consistent with the idea that ubiquitination of MutYH affects the proteasomal degradation of MutYH all lysine point mutants showed stabilization on the protein level if transfected in HEK293T cells (Figure 4 C and D). Strikingly, the 5R mutant showed a more pronounced increase in protein level (5-fold compared to WT) than the single mutants (1.5 – 2.5 fold) (Figure 4 C and D), leading to the assumption that all five lysine residues appear to contribute to the ubiquitination of MutYH.

Ubiquitination Deficient MutYH is Preferentially Bound to Chromatin.

Besides protein stability, ubiquitination can also influence the intracellular localization of a protein (22). To figure out whether the amount of ubiquitinated MutYH determines the localization of the protein within the cell, cell fractionation experiments were performed (Figure 5 A). HEK293T cells transfected with either the MutYH WT or the 5R mutant were fractionated into cytoplasmic, nuclear and chromatin bound fractions. We observed that the ubiquitination deficient mutant of MutYH was enriched in the chromatin bound fraction with respect to the WT (Figure 5 B), while the amount of MutYH in the cytoplasm remained comparable (Figure 5 C). This effect could be further confirmed by using an immunocytochemistry approach. HeLa cells were transfected with the HA-tagged MutYH WT or the 5R mutant and stained for HA and the nuclear marker DAPI (4',6-Diamidino-2-Phenylindole). The result clearly showed that the MutYH WT was exclusively localized in the cytoplasm while the 5R mutant was also present in the nucleus to a substantial amount (Figure 5 D). Taken together these results clearly showed that ubiquitination of MutYH not only influences its turnover but also changed its subcellular localization.

ROS Treatment Increases the 8-oxo-G Levels and the Mutation Frequency in A2780 Ovarian Cancer Cells.

MutYH plays an important role in the repair of 8-oxo-G thereby preventing the onset of deleterious mutations (23,24). Consequently we expected changes in the mutation frequency to happen according to the amount of MutYH present in the cells. To stimulate the formation of 8-oxo-G lesions A2780 cells, either transfected with a Mule construct (Mule ΔN2474) or Mule siRNA, were treated with $KBrO_3$. From Figure 6 A it is evident that the 8-oxo-G levels in the cells increased upon treatment with increasing amounts of $KBrO_3$, as determined in a mass spectrometry based assay. The observed levels of 8-oxo-G are in accordance with the numbers reported in literature (data not shown), The results showed a clear tendency that the treatment with $KBrO_3$ resulted in a higher oxidative burden for the cell.

We have already shown above the dependency of MutYH levels on the amount of Mule (Figure 2). Such relationship was also confirmed in the A2780 cell system. Figure 6 B clearly shows an increase of endogenous MutYH protein levels upon KD of Mule and, conversely, a decrease upon Mule

overexpression. The mutation frequency at the HPRT locus in these cells was also analyzed by selection with 6-Thioguanine (6-TG), a toxic guanine analogue. Mule KD cells had a higher MutYH level (Figure 6 B) and a lower incidence of mutations in the HPRT locus (Figure 6 C). The opposite was observed for the Mule (Mule ΔN2474) overexpressing cells, showing a decrease in MutYH level with respect to the control (Figure 6 B and C).

These data confirmed the hypothesis that the Mule dependent regulation of MutYH influences the susceptibility of cells to mutagenesis caused by exposure to oxidative stress. This clearly indicated that the regulation of MutYH has a direct impact on the mutation frequency in cells, underlining again the importance of a tight and controlled regulation of the BER machinery.

DISCUSSION

In this work we provide evidence that ubiquitination regulates not only the steady state levels of MutYH, but also its subcellular localization. We identify Mule to be the E3 ubiquitin ligase responsible for the modification of MutYH. Mule mainly appears to monoubiquitinate MutYH. Whether other E3 ubiquitin ligases are involved in the polyubiquitination and proteasomal degradation remains so far elusive. Consistent with the predicted role of Mule in the proteasomal degradation of MutYH, we find the MutYH protein levels to be reversely correlated with the levels of Mule present in HEK 293T cells. Five lysine residues could be identified as targets for ubiquitination by Mule. The clear stabilization of the ubiquitination-deficient MutYH mutants confirms the role that they are playing in the proteasomal degradation of the protein.

The enzymatic activity of MutYH needs to be directed to the nucleus in order to act upon the newly synthesized strand immediately after replication, to prevent the formation of deleterious G:C->T:A transversion mutations (26,27). Our data implicate that the ubiquitination of MutYH plays an important role in enrichment of MutYH on chromatin. We observed that mutation of the ubiquitination sites result in strong accumulation of MutYH in the chromatin bound cell fraction, respectively in the nucleus as shown by immunofluorescence. We previously found that the recruitment of Pol λ to chromatin is mediated by interplay of ubiquitination and phosphorylation as well as by formation of active repair complexes on chromatin (10,28,29). Comprising the recent data it seems very likely that MutYH might coordinate the MutYH/Pol λ dependent LP-BER pathway (17). Whether, like for Pol λ, it is an orchestration of different PTM adjusting the subcellular localization and protein levels of MutYH remains to be elucidated.

As noted in the literature a cell contains 1000-7000 8-oxo-G as a steady state level that can be repaired any time (25). MutYH has an important role in the repair of these lesions, thus mis-regulation or mutations of the enzyme cause an increased mutation frequency (8,30). In accordance we see differences in the mutation frequency depending on the levels of MutYH. In general the mutation frequency at the HPRT locus increases upon $KBrO_3$ treatment of A2780 cells. Further, the data suggest that cells with elevated MutYH levels, due to KD of Mule, are better able to cope with oxidative stress than cells with a lower amount of MutYH, upon Mule overexpression.

In conclusion, the data presented here underline again the importance of a tight and controlled regulation of BER, since already small differences in protein levels can have a pivotal effect on the genome integrity. In line with previous results, showing BER enzymes to be regulated by PTM to ensure a faithful repair of 8-oxo-G lesions, we observed for the first time a similar regulation for the essential MutYH DNA glycosylase.

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FIGURES LEGENDS

Figure 1. MutYH is ubiquitinated *in vitro* and *in vivo* by the E3 ubiquitin ligase Mule.

(A) *In vitro* ubiquitination of MutYH by the recombinant and purified HECT domain of Mule. (B) Co-transfection of Flag-Mule ΔN2474 and HA-MutYH into HEK293T cells. The cells were treated with 10

mM MG132 for 16 h prior to harvest. (C) Co-transfection of Flag-Mule ΔN2474 C4341A and HA-MutYH into HEK293T cells. The cells were treated with 10 mM MG132 for 16 h prior to harvest. (D) Mule KD in HEK293T cells, analyzed by Western blotting. 5% of total cell extracts were loaded as input from E. (E) Immunoprecipitation of Flag-Mule ΔN2474, co-transfected with HA-Ubiquitin. The cells were treated with Mule siRNA (efficiency confirmed in D) or scr siRNA as indicated and the samples were analyzed by Western blotting. (F) Quantification of E. The amount of ubiquitinated MutYH was normalized to the amount of unmodified Flag-MutYH bound to the beads.

Figure 2. MutYH protein levels depend on the amount of the E3 ubiquitin ligase Mule. (A) The effect of Mule KD in HEK293T cells, analyzed by Western blotting. (B) Quantification of protein levels shown in A. The protein levels of MutYH were normalized to tubulin. (C) Quantification of MutYH mRNA extracted from the samples shown in A. The MutYH mRNA was normalized to the levels of L28 mRNA. (D) The effect of Flag-Mule ΔN2474 overexpression in HEK 293T cells, analyzed by Western blotting. (E) Quantification of protein levels shown in D. The protein levels of MutYH were normalized to tubulin. (F) Quantification of the MutYH mRNA extracted from the samples shown in D. The MutYH mRNA was normalized to the levels of L28 mRNA.

Figure 3. MutYH and Mule physically interact. (A) GST-pulldown of GST-MutYH with His-HECT. The recombinant and purified proteins were incubated with GST-beads. (B) Pulldown of Flag-HECT with GST-MutYH. Flag AB coupled beads were incubated with cell extracts containing Flag-HECT, washed and incubated with the recombinant and purified GST-MutYH. (C) Co-immunoprecipitation of Flag-Mule ΔN2474 with HA-MutYH. Whole cell extracts containing the overexpressed proteins were incubated with Flag AB coupled to beads. (C) Co-immunoprecipitation of Flag-Mule ΔN2474 with HA-MutYH. Whole cell extracts containing the overexpressed proteins were incubated with HA AB coupled to beads. The band marked with an asterisk (*) corresponds to the MutYH signal, the lower band is an unspecific signal from IgG.

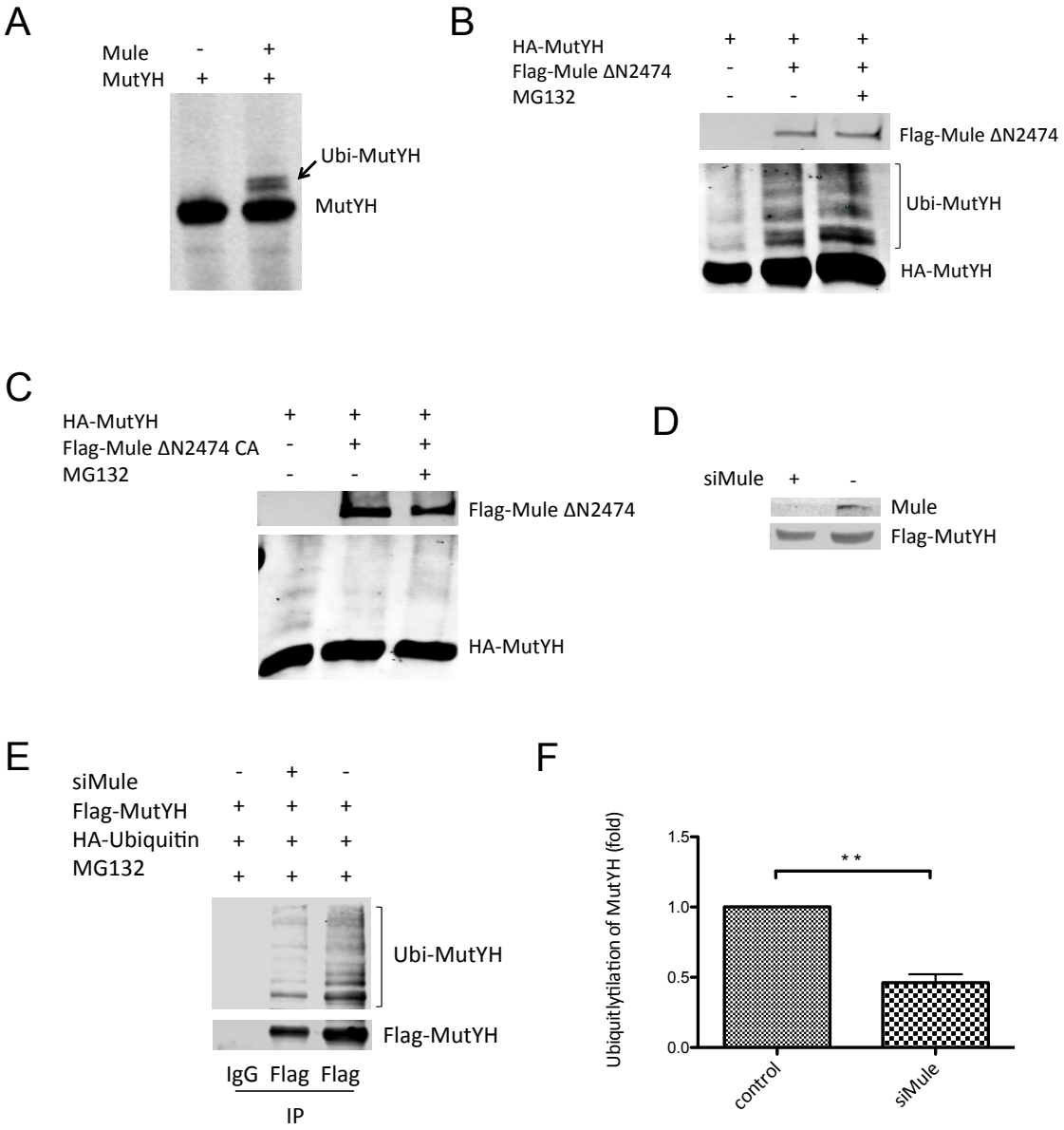
Figure 4. MutYH is ubiquitinated between amino-acids 475-535 and mutation of the five lysine residues in this part of the protein stabilize MutYH on the protein level. (A) *In vitro* ubiquitination of different recombinant and purified MutYH deletion constructs, as depicted in the upper part. (B) Amino-acid sequence of MutYH between position 475 and 535. Highlighted are the K residues that were mutated into R. (C) Transfection of the MutYH point mutant constructs (as indicated) into HEK 293T cells, analyzed by Western blotting. (D) Normalization of the mutant MutYH protein level as shown in C to the mRNA level of the respective expression construct. The protein levels were normalized to actin and the mRNA level to L28 mRNA.

Figure 5. Ubiquitination deficient MutYH is preferentially bound to chromatin. (A) Transfection of MutYH WT or 5R mutant into HEK 293T cells. The cells were fractionated and analyzed by Western blotting. (B) Quantification of the MutYH protein level in the chromatin bound fraction shown in A. The protein levels were normalized to the histone H3. (C) Quantification of the MutYH protein level in

the cytoplasmic fraction shown in A. The protein levels were normalized to the tubulin. (D) Fluorescence microscope images of HeLa cells transfected with MutYH WT or the 5R mutant. The cells were fixed and stained with HA AB and DAPI.

Figure 6. ROS treatment increases the 8-oxo-G level and the mutation frequency in A2780 ovarian cancer cells. (A) A2780 cells were treated with increasing amounts of $KBrO_3$, the DNA was isolated and analyzed for 8-oxo-G as described in Material and Methods. (B) Effect of Mule KD and Flag-Mule ΔN2474 overexpression in A2780 cells, analyzed by Western blotting. (C) Mutation frequency at the HPRT locus in A2780 cells, either treated with Mule siRNA or transfected with Flag-Mule ΔN2474 and, as indicated, incubated with increasing amounts of $KBrO_3$.

Figure 1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MutYH
B
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Ubi-MutYH
HA-MutYH
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HA-MutYH + + +
Flag-Mule ΔN2474 CA - + +
MG132 - - +
Flag-Mule ΔN2474
HA-MutYH
D
siMule + -
Mule
Flag-MutYH
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siMule - + -
Flag-MutYH + + +
HA-Ubiquitin + + +
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Figure 2

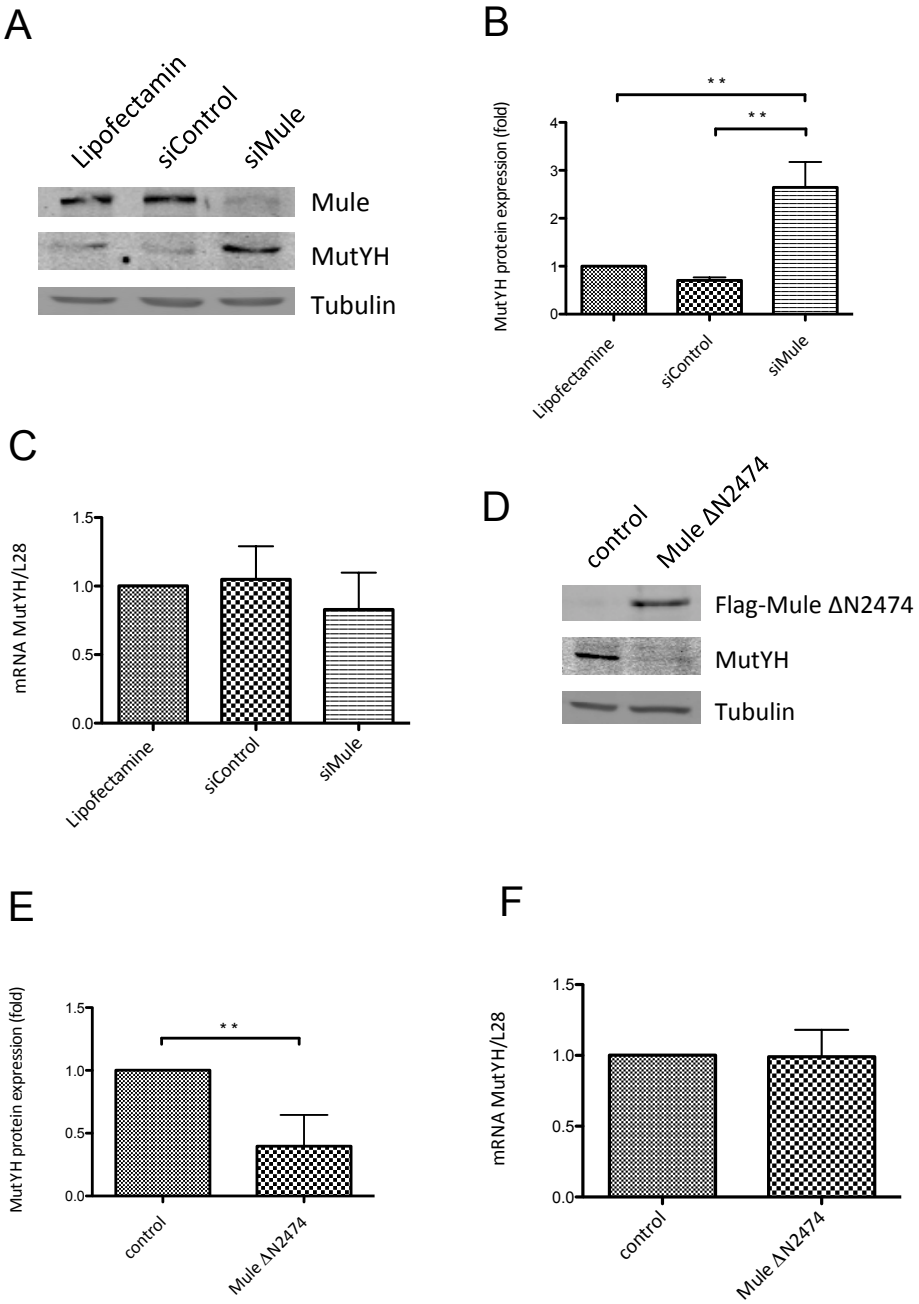
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Figure 3

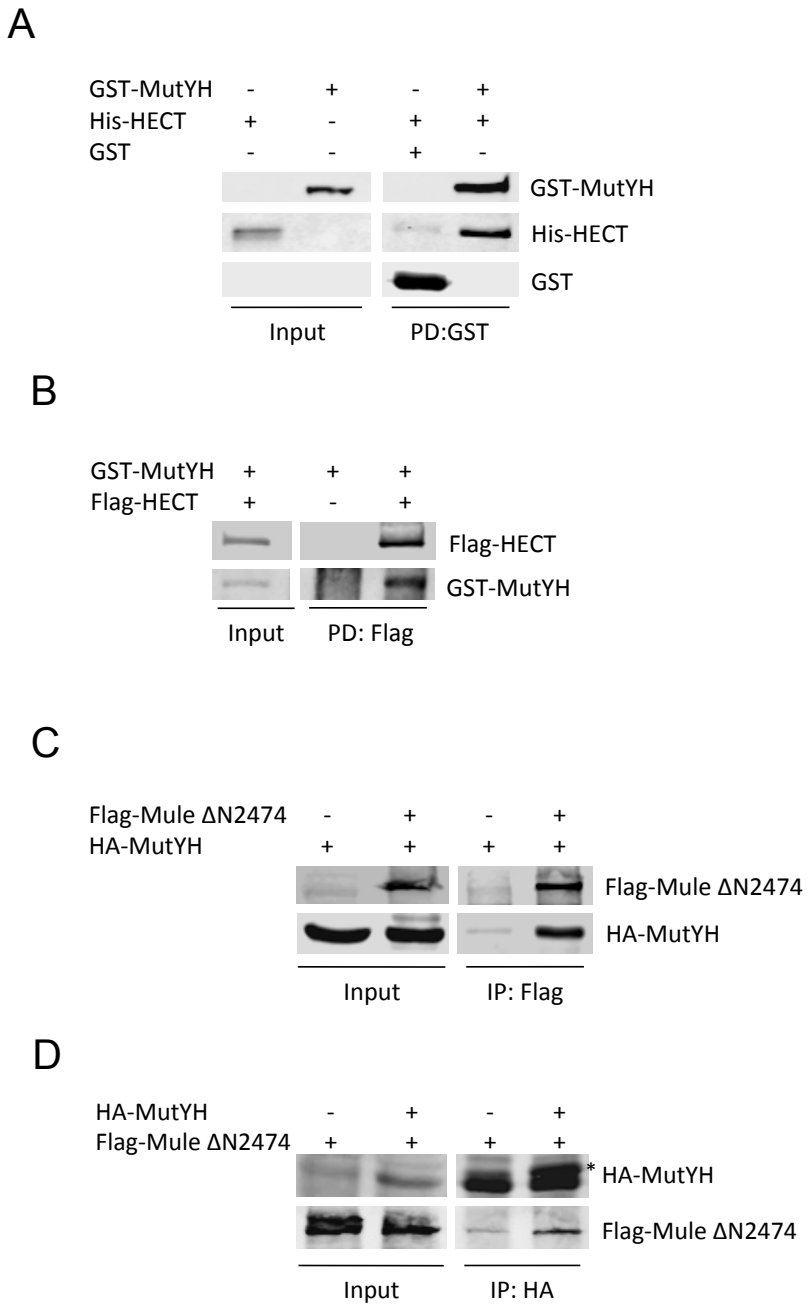

A
GST-MutYH - + - +
His-HECT + - + +
GST - - + -
GST-MutYH
His-HECT
GST
Input
PD:GST
B
GST-MutYH + + +
Flag-HECT + - +
Flag-HECT
GST-MutYH
Input
PD: Flag
C
Flag-Mule ΔN2474 - + - +
HA-MutYH + + + +
Flag-Mule ΔN2474
HA-MutYH
Input
IP: Flag
D
HA-MutYH - + - +
Flag-Mule ΔN2474 + + + +
* HA-MutYH
Flag-Mule ΔN2474
Input
IP: HA

Figure 4

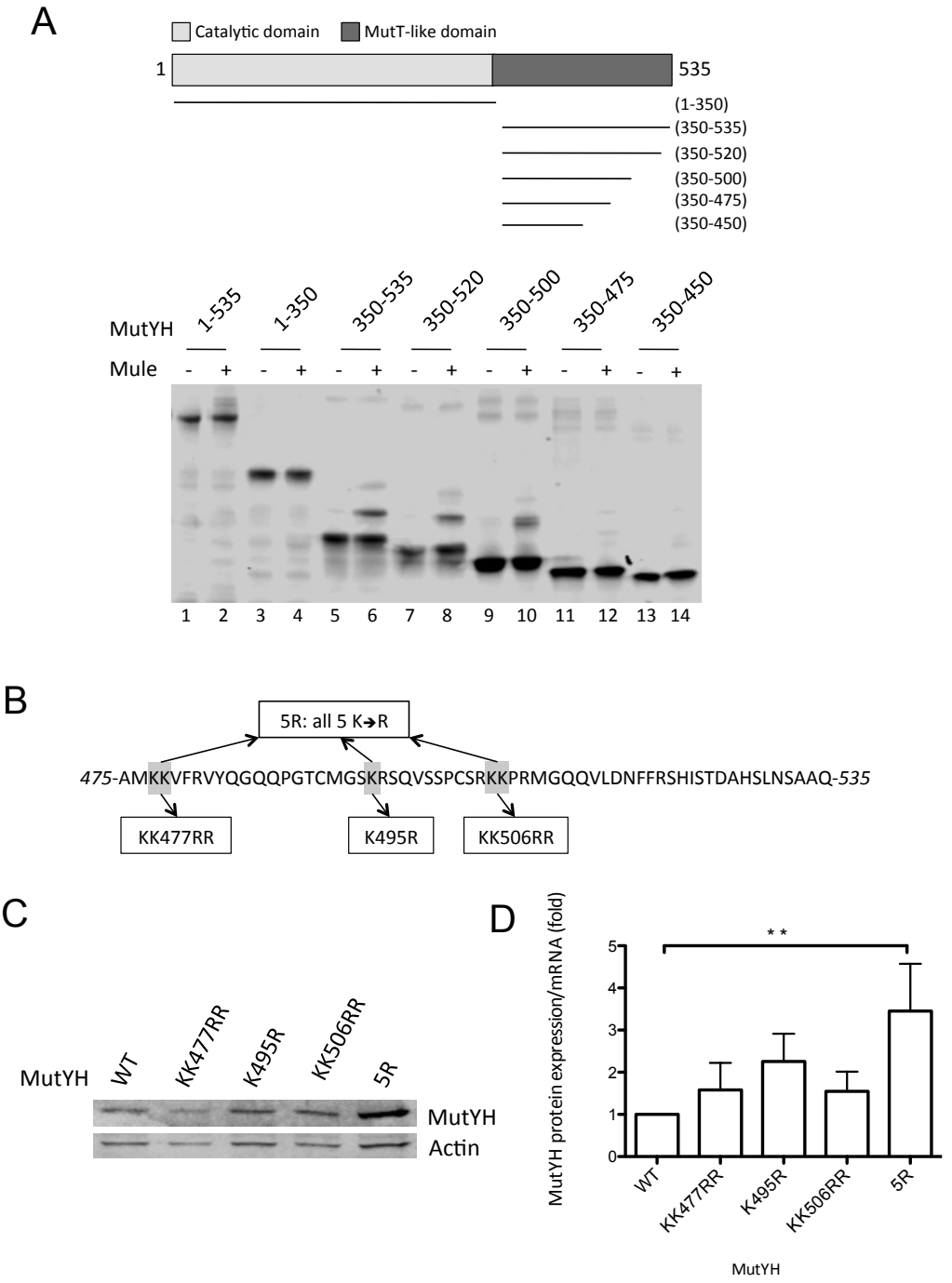

A
Catalytic domain
MutT-like domain
1
535
(1-350)
(350-535)
(350-520)
(350-500)
(350-475)
(350-450)
MutYH
1-535
1-350
350-535
350-520
350-500
350-475
350-450
Mule
- + - + - + - + - + - + - +
1 2 3 4 5 6 7 8 9 10 11 12 13 14
B
5R: all 5 K→R
475-AMKKVFRVYQGQQPGTCMGSKRSQVSSPCSRKKPRMGQQVLDNFFRSHISTDAHSLNSAAQ-535
KK477RR
K495R
KK506RR
C
MutYH
WT
KK477RR
K495R
KK506RR
5R
MutYH
Actin
D
MutYH protein expression/mRNA (fold)
* *
0
1
2
3
4
5
WT
KK477RR
K495R
KK506RR
5R
MutYH

Figure 5

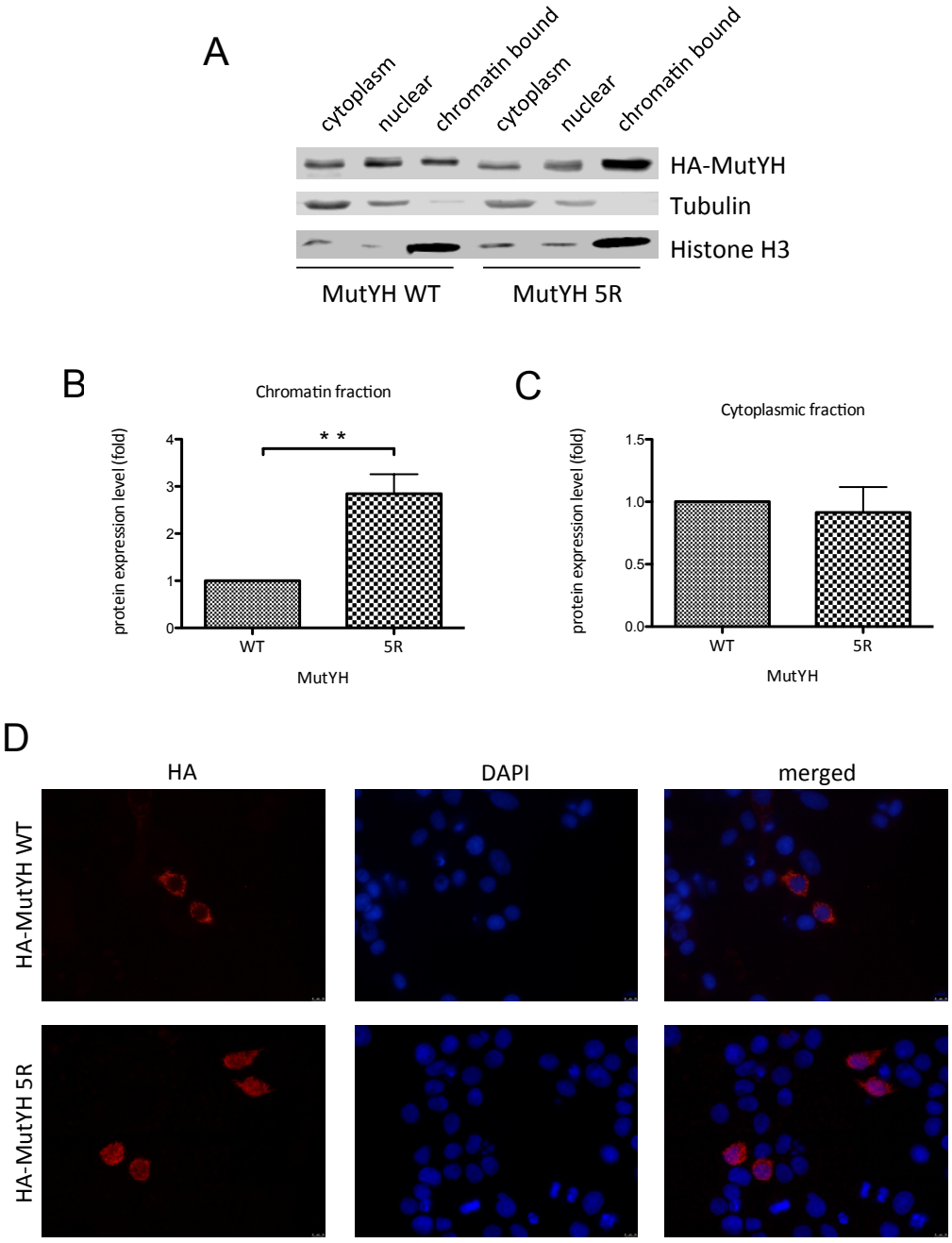
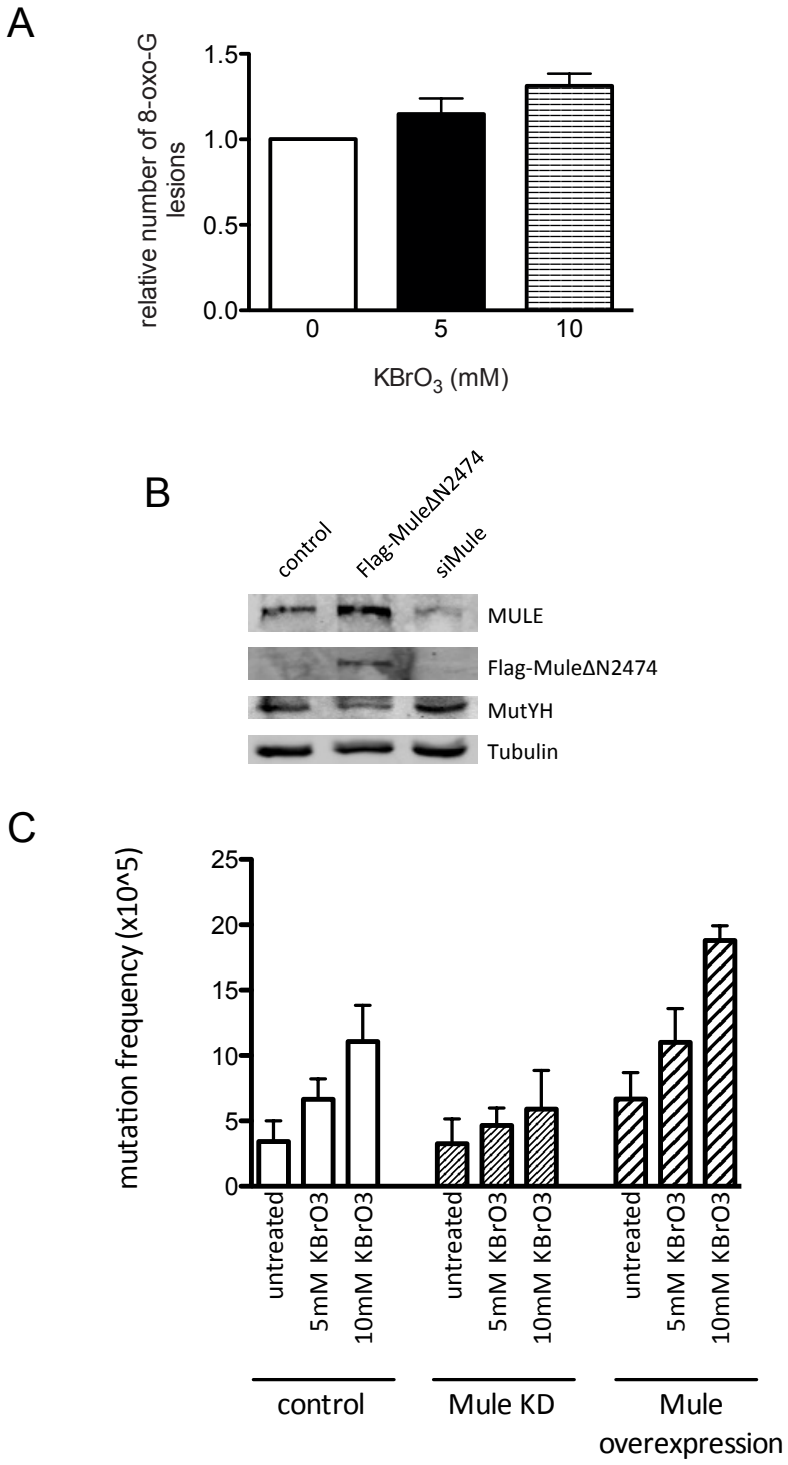

A
cytoplasm
nuclear
chromatin bound
cytoplasm
nuclear
chromatin bound
HA-MutYH
Tubulin
Histone H3
MutYH WT
MutYH 5R
B
Chromatin fraction
protein expression level (fold)
* *
WT
5R
MutYH
C
Cytoplasmic fraction
protein expression level (fold)
WT
5R
MutYH
D
HA
DAPI
merged
HA-MutYH WT
HA-MutYH 5R

Figure 6


A
relative number of 8-oxo-G lesions
1.5
1.0
0.5
0.0
0
5
10
KBrO3 (mM)
B
control
Flag-MuleΔN2474
siMule
MULE
Flag-MuleΔN2474
MutYH
Tubulin
C
mutation frequency (x10^5)
25
20
15
10
5
0
untreated
5mM KBrO3
10mM KBrO3
untreated
5mM KBrO3
10mM KBrO3
untreated
5mM KBrO3
10mM KBrO3
control
Mule KD
Mule overexpression

5. Unpublished data

5.1. Interaction studies of DNA polymerase λ

5.1.1. Interaction of DNA polymerase λ and Ogg1 DNA glycosylase

DNA Pol λ was shown to interact with many enzymes involved in DNA damage repair, like PCNA (135) and MutYH (40). To investigate whether Ogg1 is also a binding partner of DNA Pol λ co-immunoprecipitation experiments were performed. Whole cell extracts were generated either out of HEK293T or HeLa cells and endogenous DNA Pol λ was coupled to beads using DNA Pol λ antibody (AB). As shown in Figure 10 Ogg1 was co-precipitated in both cell lines confirming an interaction between the two proteins. Whether the interaction is direct or rather mediated by another binding partner remains was not tested.

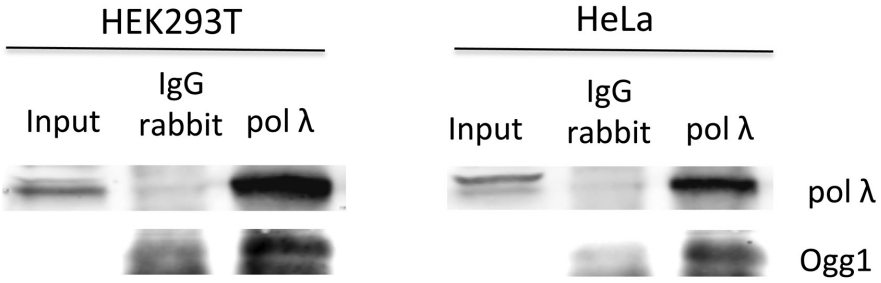


Figure 10: Interaction of DNA Pol λ and Ogg1.
DNA Pol λ AB coupled beads were used to immunoprecipitate DNA Pol λ from either HEK293T or HeLa whole cell extract. Binding of Ogg1 was analyzed by Western blotting.

Since many protein interactions are modulated by PTMs the influence of phosphorylation on the interaction between DNA Pol λ and Ogg1 was addressed. Co-immunoprecipitation experiments from HEK293T whole cell extracts were performed either in the presence or absence of phosphatase inhibitors. The inhibition of phosphatase activity in the cell extracts led to a dramatic decrease in the binding of Ogg1 to DNA Pol λ, suggesting a dependency on phosphorylation (Figure 11 A). To confirm this result in another approach the Pol λ coupled beads were incubated with calf intestine phosphatase (CIP) prior to analysis of Ogg1 binding. As shown in Figure 11 A and B also the removal of phosphate residues on the bound proteins influenced the interaction. Consistent with the first result the interaction between DNA Pol λ and Ogg1 appears to be stronger in the absence of phosphorylation.

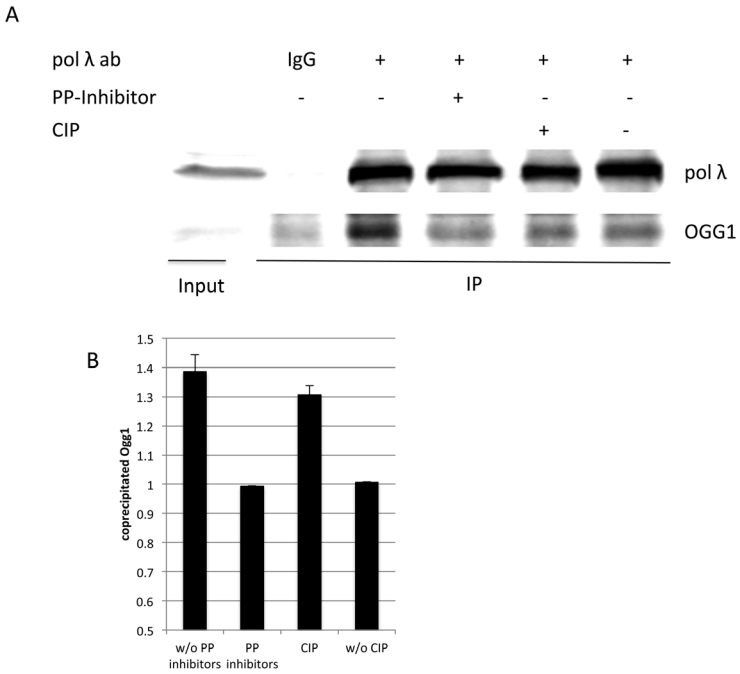


Figure 11: The interaction between DNA Pol λ and Ogg1 is phosphorylation dependent.
(A) Coimmunprecipitation of DNA Pol λ and Ogg1 from HEK293T whole cell extract. (B) Quantification of the data shown in A.

Discussion

DNA Pol λ plays an important role in the repair of 8-oxo-G lesions that can arise from a variety of endogenous and exogenous sources. In contrast to the replicative Pols DNA Pol λ is able to incorporate the correct C opposite a 8-oxo-G lesion thereby preventing the formation of G:C->T:A transversion mutations (99). Subsequently the resulting 8-oxo-G:C pair is a substrate for the DNA glycosylase Ogg1 that can excise the lesion. An interaction between DNA Pol λ and Ogg1 might facilitate the repair of the lesion by directing the executing glycosylase to the site of damage. DNA repair processes need to be tightly regulated to prevent any unintentional action. The binding of DNA Pol λ to Ogg1 seems to be regulated by phosphorylation. The results presented here are in accordance with previous findings showing that phosphorylation of DNA Pol λ regulates the steady state levels of the protein by interfering with the ubiquitination of the protein (67). Taken together these results show that BER is regulated by interplay between different PTM modulating not only the presence of repair complexes on chromatin but also the interaction between proteins.

To gain further insides in the regulation of BER it would be of great interest to investigate which enzymes under which conditions are involved in the phosphorylation of DNA Pol λ.

5.1.2. Interaction of DNA polymerase λ and MutYH DNA glycosylase

MutYH was shown to be an interaction partner of DNA Pol λ (40), but so far it was not determined which part of MutYH is involved in the binding. To address this question further different GST-tagged deletion constructs of MutYH were cloned, expressed and purified (Figure 12).

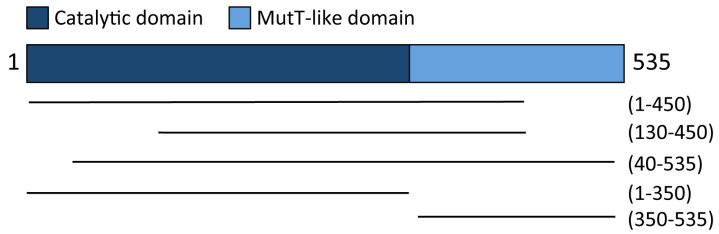


Figure 12: Scheme of MutYH deletion constructs used for pulldown experiments.

The MutYH constructs were used to perform GST-pulldown experiments with recombinant and purified DNA Pol λ. As shown in Figure 13 DNA Pol λ co-precipitated with the full length MutYH (lane 8) as well as with the N-terminal part of MutYH (lane 5). An interaction could not be detected neither with a construct missing the very N-terminal 130 amino-acids (lane 6) nor with the C-terminal part of MutYH (lane 7). These results led to the conclusion that the interaction site is located within in the N-terminal 130 amino-acids of MutYH.

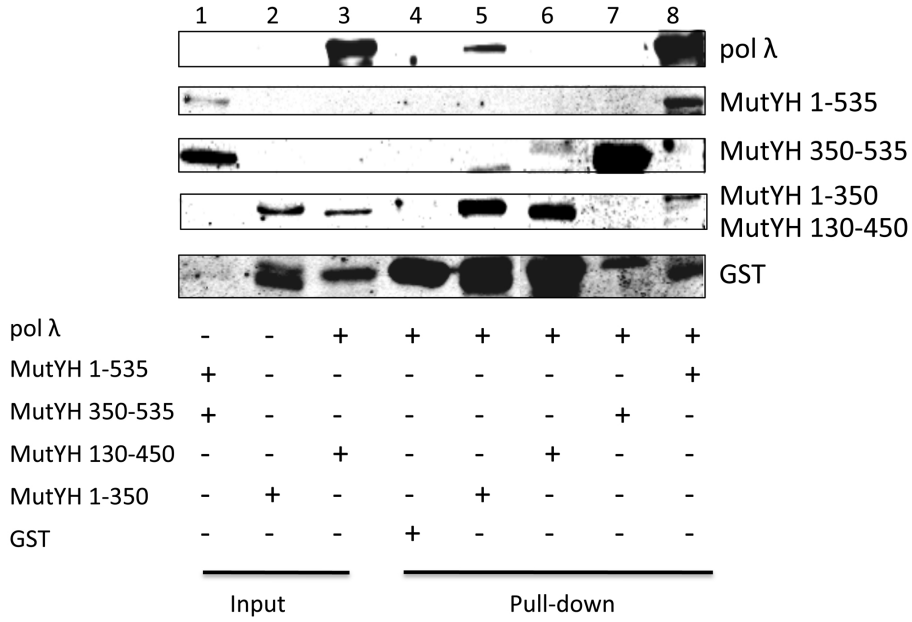


Figure 13: GST-pulldown of MutYH and DNA Pol λ.
The pulldown was performed under the conditions described in section 4.

To narrow the region involved in the interaction further down another MutYH deletion construct (40-535) was included in the experiment (Figure 14). The result in Figure 14 showed that this construct was able to bind DNA Pol λ (lane 8) leading to the conclusion that the N-terminal amino-acids 40-130 of MutYH mediated the interaction with DNA Pol λ.

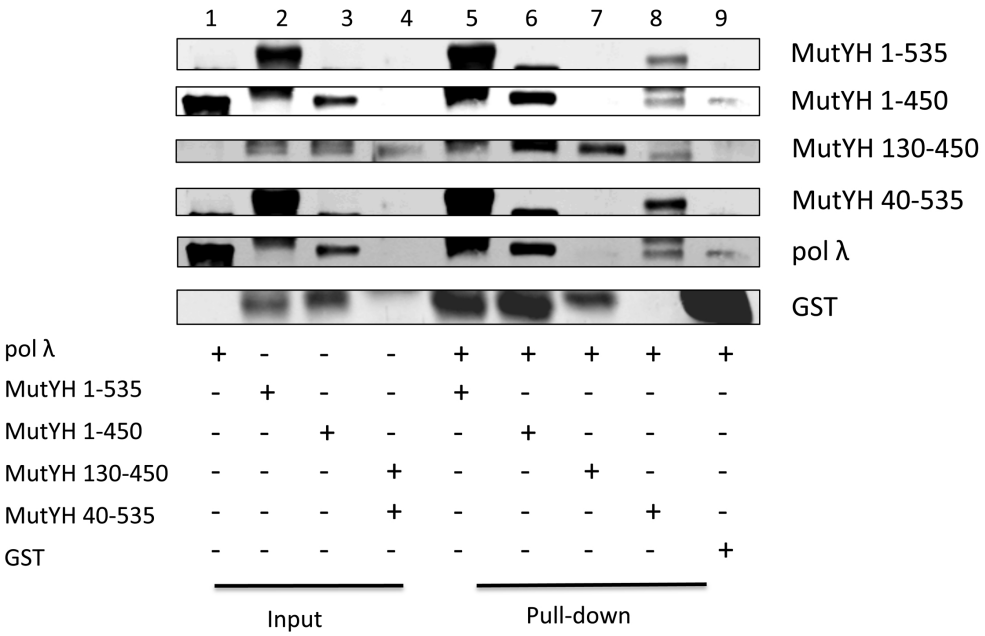


Figure 14: GST-pulldown of MutYH and DNA Pol λ.
The pulldown was performed under the conditions described in section 4.

Mutations in MutYH were shown to have an important impact on the onset of colorectal cancer. Germline mutations in many positions of MutYH were identified in patients suffering from MAP (136). Among those are two missense mutations within the region 40-130, V61E and W117R. To investigate whether these mutations influence the binding to DNA Pol λ point-mutation constructs of MutYH were tested. As can be seen in Figure 15 neither the V61E (lane 7) nor the W117R (lane 8) mutant showed a changed in the binding capacity to DNA Pol λ with respect to the MutYH WT (lane 9).

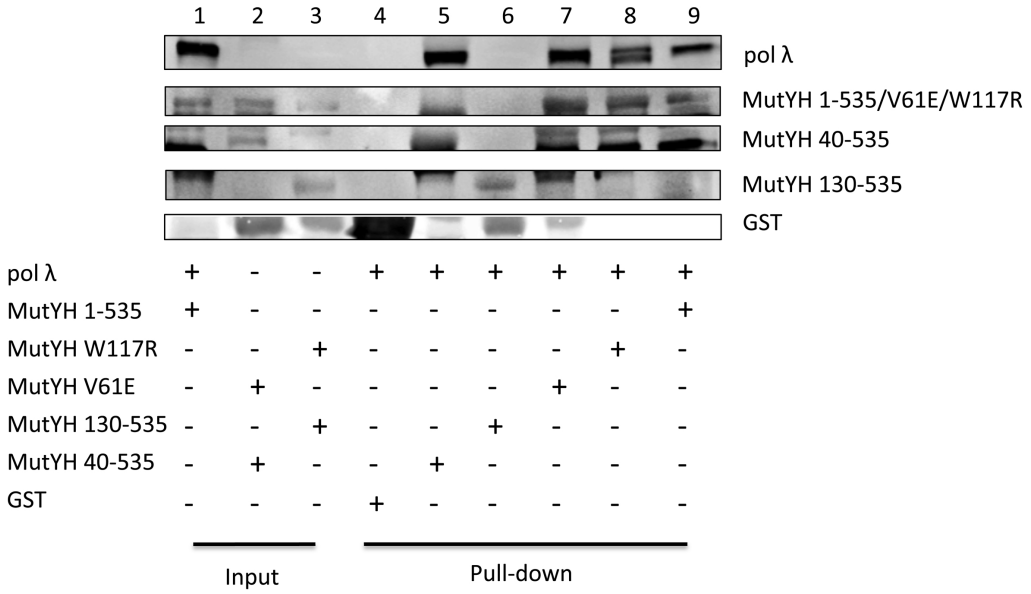


Figure 15: GST-pulldown of MutYH point mutants V61E and W117R and DNA Pol λ.
The pulldown was performed under the conditions described in section 4.

Discussion

If replication occurs before 8-oxo-G lesions are removed by Ogg1 the formation of 8-oxo-G:A mispairs is very likely. In this case MutYH can recognize and remove the mispaired A. Subsequently DNA Pol λ is able to incorporate the correct C opposite the lesion thereby giving the cell another chance to repair the lesion correctly. The interaction of DNA Pol λ and MutYH was already described in the context of the MutYH initiated LP-BER pathway (40). The results presented here confirm this interaction and specify the amino-acids 40-130 of MutYH to be essential for the binding of DNA Pol λ. Two point mutations that were identified in MAP patients are located within this region but do no seem to affect the interaction. Since the catalytically active site is located in the N-terminal part of MutYH it would be interesting to investigate whether these mutations have any impact on the glycosylase activity.

5.2. Stability of MutYH

The proteasomal degradation of many proteins is mediated by binding to other proteins or protein-complexes (123,137). MutYH is a central player of the BER and interacts with proteins involved in DNA damage repair processes (reviewed in (138)). Based on the findings reported before (section 4) it was further examined whether the proteasomal degradation of MutYH is modulated by the interaction with other proteins. A screen using siRNA against Rad1, DNA ligase 1, DNA Pol λ, APE1, PCNA, PARP1, Rad9, Hus1, Ogg1, XRCC1 and MSH6 showed that only the depletion of XRCC1 led to a dramatic reduction of MutYH on protein level (Figure 16).

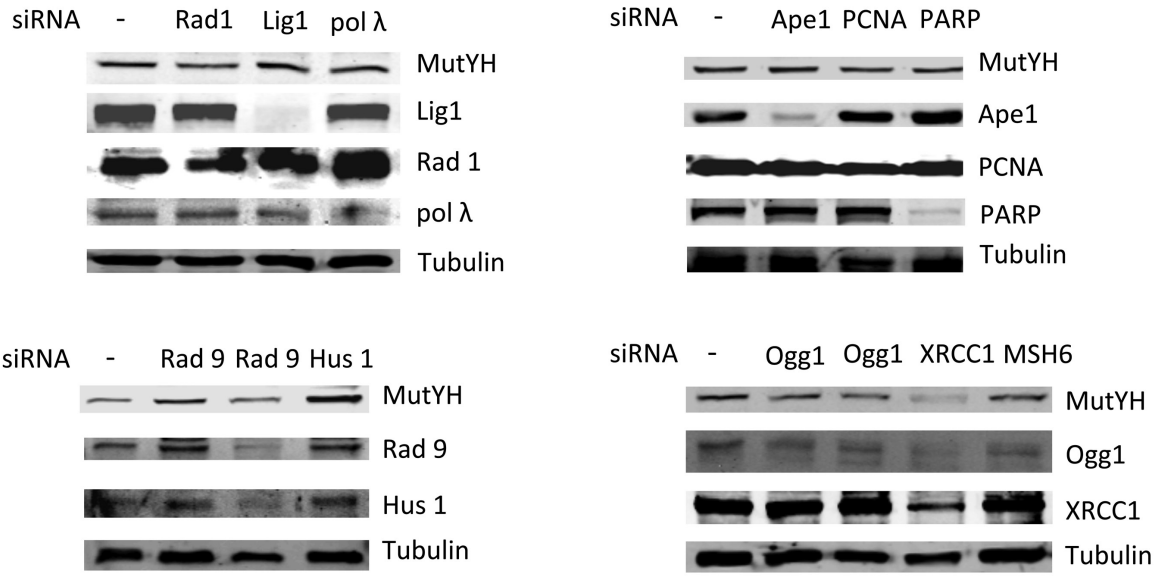


Figure 16: XRCC1 depletion causes a decrease in MutYH level.
Knock down was performed in HEK293T cells using siRNA as indicated. The experiment was performed under the conditions described in section 4. and analyzed by Western blotting.

This result could be further confirmed as shown in Figure 17 A and B. The regulation of MutYH upon XRCC1 took place on protein level since the mRNA remained almost unchanged (Figure 17 B). This result indicates that also the interaction with other proteins might contribute to the stabilization and protection of MutYH from proteasomal degradation.

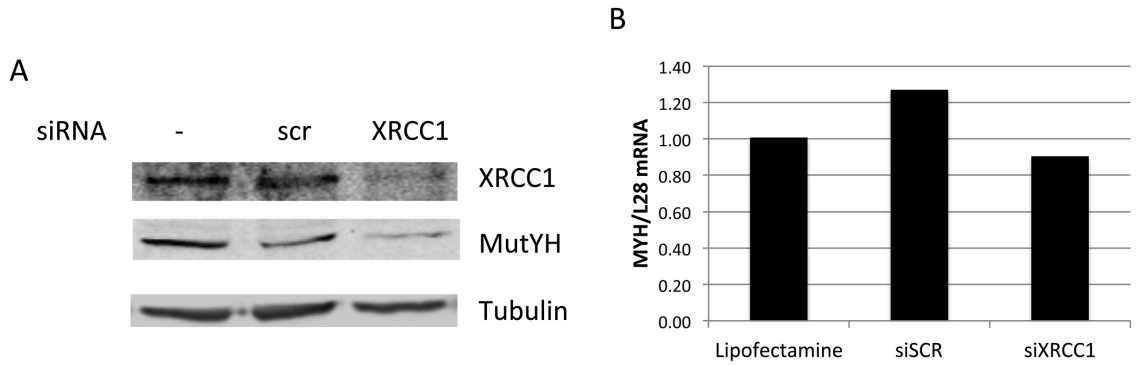


Figure 17: XRCC1 knock down causes a decrease of MutYH on protein level.
(A) XRCC1 knock down was performed under the conditions described in section 4. and analyzed by Western blotting. (B) Quantitative PCR analysis of the samples presented in A. The MutYH mRNA level were normalized to L28.

Discussion

The formation of protein complexes not only serves as functional link between enzymes but can also protect their members from proteasomal degradation. The steady state levels of DNA Pol β as well as DNA ligase III were shown to be modulated by binding to XRCC1 (123,139). The interaction with XRCC1 renders the proteins in both cases more stable and protected from proteasomal degradation. XRCC1 is an important scaffold protein able to bind AP sites and to facilitate the repair process by coordinating the executing enzymes (140). Thus it is very likely that also the susceptibility of MutYH to proteasomal degradation might be regulated in a similar manner. Further experiments would be required to show whether a direct interaction between XRCC1 and MutYH is taking place, thus explaining the stabilizing effect.

6. Summary of the main findings

In order to give a clear overview of the main findings disclosed in this thesis a short summary is given below.

- The E3 ligase Mule ubiquitinates MutYH *in vitro* and *in vivo*.
- Mule knock down leads to an increase in endogenous MutYH protein levels.
- Mule overexpression causes a decrease in MutYH protein levels.
- Mule and MutYH interact physically.
- The ubiquitination sites of MutYH are located within the amino-acids 475-535.
- The ubiquitination deficient mutant of MutYH is stabilized on protein level.
- The ubiquitination deficient mutant of MutYH is predominantly bound to chromatin.
- Treatment with $KBrO_3$ increases the 8-oxo-G levels and the mutation frequency at the HPRT locus in the ovarian cancer cells A2780.

7. Discussion

Many enzymes involved in DNA damage repair are targets for PTM that can influence the function, protein level or subcellular localization of the enzymes (141). But so far just few studies investigated how these regulatory mechanisms are cross-linked with each other. Since DNA damage can occur all the time repair enzymes are needed permanently to ensure the genomic integrity. Consistently BER enzymes are rather “fine tuned” on the steady state level than completely “shut on or of” (127).

In this thesis it is shown for the first time, how the DNA glycosylase MutYH is regulated on protein level. Mule was identified to be the E3 ligase responsible for the ubiquitination of MutYH. In an *in vitro* assay it could be shown that MutYH is a target for the modification mediated by Mule. This result could be further confirmed in whole cell extracts exhibiting that this reaction can also take place in a normal cellular context. Mule was shown to mainly monoubiquitinate its substrates what stimulates the subsequent polyubiquitination of the proteins (123,142). Therefore it would be very interesting to investigate whether MutYH is also a target of polyubiquitination stimulated by Mule and mediated by another E3 ligase. This question could be addressed by fractionation of whole cell extracts and isolation of the E3 activity containing fractions.

The levels of Mule in cells dramatically influenced the amount of MutYH protein levels while the amount of mRNA remained unchanged. These results serve as “proof of principle” that Mule is the E3 ligase modulating the MutYH levels in cells. Since Mule was shown to be regulated upstream by factors induced by oxidative stress (134) it would be interesting to analyze the effect that exposure to oxidative stress has on the protein levels of MutYH.

Mule and MutYH interact, as shown by co-immunoprecipitation performed out of whole cell extracts. To confirm a direct interaction and to exclude the binding via another scaffold protein pull-down experiments with recombinant and purified enzymes were performed with the same results.

To identify the sites of ubiquitination different deletion constructs of MutYH were used in an *in vitro* ubiquitination assay with Mule. The results identified five lysine residues located in the C-terminal part of the protein as targets for modification. Consistently all arginine point mutants of these sites were stabilized on protein level. The stabilization of the mutant having all lysine residues mutated was clearly the strongest, implicat-

ing that at least to a certain extend all five lysine residues can be ubiquitinated and replaced by each other.

Strikingly the ubiquitination deficient mutant of MutYH was shown not only to be stabilized but also to be predominantly bound to chromatin implicating that ubiquitination not only controls the protein levels of MutYH but also its activity on damaged DNA. This result is in accordance with previous publications confirming an important role for ubiquitination in the subcellular localization of proteins (118). Since the deubiquitinated MutYH is located on the chromatin it would be interesting to investigate whether the modification also affect the glycosylase activity or the DNA binding capacity.

MutYH is an important enzyme in BER and its inactivation or mutation was shown to impair the DNA damage repair and thereby to increase the mutation frequency in different tissues and cell lines (143).

Consistently with these findings the treatment of A2780 ovarian cancer cells with $KBrO_3$ increased the mutation frequency analyzed at the HPRT locus. This effect can be explained by the elevated levels of 8-oxo-G following the treatment as determined by mass spectrometry. Strikingly it was found that Mule knock down cells, showing elevated levels of MutYH, can cope better with the high levels of 8-oxo-G formation than the Mule overexpression cells with decreased MutYH levels. To follow this line it would be interesting to see how cells expressing the ubiquitination deficient mutant of MutYH behave upon exposure to oxidative stress.

Taken together the results presented in this thesis identified another regulatory mechanism that allows the cells to regulate the DNA damage repair. However a lot of effort needs to be done to gain more insights into the subtle regulation of the BER enzymes and to understand how all of these pathways interact with and influence each other to enable the cell finally to react properly to oxidative damage.

8. References

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10. Curriculum Vitae

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EDUCATION

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10/2004 - 01/2010 DIPLOMA STUDIES IN BIOCHEMISTRY
Eberhard Karls University Tübingen, Germany

- Final grade: 1.1
- Graduation: Biochemistry (grade: 1.3)
  Molecular Medicine (grade: 1.0)
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DIPLOMA THESIS

University Hospital Tübingen, Germany
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„Characterization of the Phosphorylation of the Kinesin-like Proteins KIF1C“ (grade: 1.0)

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PUBLICATIONS

Markkanen E, Dorn J, Hübscher U. MUTYH DNA glycosylase: the rationale for removing undamaged bases from the DNA. *Front Genet. 2013;4:18. doi: 10.3389/fgene.2013.00018. Epub 2013*

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